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ORIGINAL PAPER

Dawn Webber · Magali C Chauvet · Rosemary L Ryall

Proteolysis and partial dissolution of calcium oxalate: a comparative, morphological study of urinary crystals from black and white subjects

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Abstract Crystal adherence to the renal epithelium is widely regarded as a probable mechanism of stone formation. Intracrystalline proteins may provide access to the core of urinary crystals and thereby facilitate the dismantling of these crystals after their attachment to and phagocytosis by renal epithelial cells. The present study investigated the role of proteolysis and limited dissolution of urinary calcium oxalate (CaOx) crystals in South Africa's white and black populations with a view to understanding the remarkably low stone incidence in the black population compared with the whites. CaOx crystals were precipitated from filtered urine or ultrafiltered urine dosed with an intracrystalline protein, urinary prothrombin fragment 1 (UPTF1), isolated from white and black subjects. The crystals were fractured and subjected to proteolysis and/or limited dissolution before examination using field emission scanning electron microscopy (FESEM). FESEM data showed that CaOx crystals from white and black subjects were eroded by treatment with proteases. Cathepsin D caused the most significant crystal erosion, and more noticeable degradation of CaOx monohydrate (COM) crystals compared to CaOx dihydrate (COD). Limited dissolution studies showed the unique ultrastructures and fragmentation processes of COM and COD crystals. COM crystals appeared to be more susceptible to degradation and dissolution than CODs. Since COMs are predominant in blacks, compared with COD crystals from whites, it is speculated that the lower stone rate amongst South African blacks might be attributed partly to their more efficient destruction of retained COM crystals.

Keywords Calcium oxalate monohydrate · Calcium oxalate dihydrate · Proteolysis · Dissolution · Urinary prothrombin fragment 1

D. Webber (✉)
Department of Chemistry,
University of Cape Town,
Private Bag, Rondebosch 7701, South Africa
E-mail: dawn@science.uct.ac.za
Tel.: +27-21-6502534
Fax: +27-21-6867647

M. C Chauvet · R. L Ryall
Department of Surgery, Flinders Medical Centre
and Flinders University, Bedford Park,
South Australia 5042, Australia

Introduction

It is widely acknowledged that attachment of crystals to cells within the nephron is an indispensable forerunner to stone formation. However, although numerous studies have shown that calcium oxalate (CaOx) crystals bind avidly to cultured cells [1, 2 for reviews], the same studies have also shown that adherent crystals are phagocytosed and destroyed within lysosomal inclusion bodies [2] or by macrophages [1–4]. Therefore, crystal attachment appears to be an essential step in both stone formation and stone prevention, a paradox whose explanation will come only from studies identifying factors that influence crystal binding and subsequent destruction within the nephron.

Although the surface properties of a crystal will obviously affect its binding affinity for a cell, those properties will inevitably be influenced by the type and amount of macromolecules present in the surrounding urine or on the cell membrane. At the present time, evidence that urinary macromolecules modulate the attachment of crystals to tubular cells is contradictory. For example, a report that prior exposure of COM crystals to osteopontin (OPN) blocked the deposition of crystals to cultured renal epithelial cells [5] was disputed by other findings claiming that the protein promotes crystal adhesion [6, 7]. Further, Ebisuno et al. [8] showed that adherence of COM crystals to MDCK cells is prevented by prior exposure to human urine, while Romijn et al. [9] reported that urine had no effect. The role of surface macromolecules in crystal-cell

attachment is therefore far from clear. However, not all macromolecules associated with crystals are bound only to the surface. Selected urinary proteins are located within the mineral bulk, and they have the potential to influence the intracellular fate of their crystal hosts after they have been phagocytosed by cells to which they have attached.

There is both qualitative [10, 11] and quantitative [12] evidence that certain urinary proteins associated with the crystal matrix are in fact intracrystalline. They are intimately associated with the mineral constituent of CaOx crystals [10, 13], being distributed in channels throughout the mineral phase. Thus, it has been hypothesised that in addition to their role as inhibitors of crystallisation, some proteins may also facilitate the dismantling of urinary crystals by providing proteases with access to their protein core [10, 11]. Urinary prothrombin fragment 1 (UPTF1) is the principal protein in the organic matrix of CaOx monohydrate (COM) crystals [14, 15]. Thus, in addition to inhibiting CaOx crystallisation [16, 17], the protein may also help to prevent stones by assisting the intracellular comminution and dissolution of crystals after their attachment to and phagocytosis by renal epithelial cells. This notion is supported by the observation that urinary crystals rich in protein are susceptible to protease treatment whereas crystals composed of pure mineral are not [10, 11]. Moreover, incubation of CaOx crystals generated in human urine containing proteins causes pitting and erosion, which is not seen in the presence of a protease inhibitor cocktail or with crystals precipitated from ultrafiltered urine, demonstrating conclusively that they are susceptible to attack by endogenous proteases occurring in normal, healthy urine [18]. Thus, in vivo, crystals containing proteins will be more vulnerable to dissolution within the acidic environment of cellular lysosomes (pH ~5), as well to the proteases produced by these organelles.

The present study was undertaken to investigate the possibility that crystal proteolysis and dissolution might explain the discrepancy between the rates of stone formation in the South African population. The incidence of urolithiasis in South Africa's white population is similar to that of the western world, i.e. 12%. However, in the black population it is extremely rare [19, 20]. The juxtaposition of two groups, one stone-prone and the other virtually stone-free, within the same geographical region, provides a valuable model for the study of stone formation.

Thus, the objective of the present study was to investigate the role of proteolysis and limited dissolution of urinary CaOx crystals in South Africa's white and black populations. Furthermore, since blacks tend to excrete a larger proportion of COM crystals than whites, whose urines characteristically contain COD crystals [15], a comparison of the susceptibility of COM and COD to proteolysis and partial dissolution was undertaken.

Materials and methods

Isolation and purification of UPTF1

UPTF1, which migrated as a single band on SDS-PAGE, was isolated from urine samples collected from black and white subjects as previously described [17].

Urine collection and treatment

Urine samples (24 h) were collected with a boric acid preservative from ten healthy males (five whites, five blacks) on their free, unrestricted diets. Equal volumes of each urine were pooled according to population group and in accordance with the recommendations of a study for the optimum pooling of samples [21]. The pooled urines were filtered (0.75 μm prefilter, 0.45 μm nitrocellulose, 0.22 μm Millipore) and a portion of each was retained as a control sample. In a separate experiment, additional urine samples collected from the same subjects were filtered as described, and then ultrafiltered through a membrane with a 10 kDa cut-off (Millipore) to remove all macromolecules larger than 10 kDa, including UPTF1. The ultrafiltered urine was divided into three aliquots and either left unaltered, or dosed with either UPTF1 derived from white (WF1) or black subjects (BF1) at final concentrations of 1.25 mg/l (Table 1).

Preparation of CaOx urinary crystals

The CaOx metastable limits (MSL) of the pooled, filtered urines were determined according to Ryall et al. [22]. Since neither ultrafiltration nor the addition of UPTF1 alters the MSL [16, 23], equal sodium oxalate loads were added to all four urine samples from each population group. Crystallisation was induced by the addition of sodium oxalate loads (30 μmol/l in excess of MSL) at 0 and 1 h. The samples were incubated at 37°C in a shaking water bath (100 rpm) and after a total incubation period of 2 h, the crystals were collected by filtration (0.22 μm). The crystals were washed briefly with 0.1 mol/l NaOH, thoroughly with distilled water, and then dried.

Table 1 Abbreviations used to denote CaOx crystals precipitated from the urines of white and black subjects

Urine sample	White subjects	Black subjects
Filtered urine	WF	BF
Ultrafiltered urine	WUF	BUF
Ultrafiltered urine dosed with WF1	WUF + WF1	BUF + WF1
Ultrafiltered urine dosed with BF1	WUF + BF1	BUF + BF1

In a separate experiment, additional urines from five white and five black subjects were collected and ultrafiltered, and CaOx crystals were prepared as described above: these crystals served as controls. Crystals prepared from the four urine samples of each population group are henceforth referred to by the abbreviations shown in Table 1.

Western blotting

Portions of crystals were demineralised in 0.25 mol/l EDTA (pH 8.0) (100 ml/g), desalted using a P2 BioGel (BioRad Laboratories, Richmond, USA) desalting column and the protein fraction was freeze-dried. The proteins were resolved by SDS-PAGE on 15% polyacrylamide gels and transferred onto nitrocellulose membranes for immunodetection. One half of the membrane was equilibrated in a 3:1000 dilution of a monoclonal prothrombin antibody, B19–1 IgG [24], and the other was equilibrated in a 1:2500 dilution of rat anti-human osteopontin (OPN) IgG (Chemicon, Temecula, USA). The secondary antibodies used were goat anti-mouse IgG HRP conjugate (1:250 dilution, BioRad Laboratories, Richmond, USA) and goat anti-rat IgG HRP conjugate (3:4000 dilution, Chemicon) for the prothrombin and OPN blots, respectively. Membranes were developed in a solution of 3,3′′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St Louis, USA).

Treatment of crystals

Prior to treatment, portions of the crystals were placed on a glass slide and fractured using a metal spatula (fracturing was confirmed by light microscopy). Crystals washed with water alone served as controls. The crystals were then treated in one of three ways, as follows.

Proteolysis

Crystals (WF and BF, 0.25 mg each) were incubated at 37°C for 16 h with agitation (100 rpm) in 0.5 ml of a saturated solution of CaOx containing 10.0 mmol/l NaCl, 12.5 mmol/l Tris, pH 6.0, and 2 units/ml of cathepsin D (Sigma Chemical), thrombin (CSL Limited, Victoria, Australia) or Proteinase K (Roche Diagnostics, Castle Hill, Australia). The saturated CaOx solution was prepared by vigorously stirring 3 g of solid COM (BDH Chemicals Poole, England) in 2 l of distilled water at 90°C for 15 min and then immediately transferring the suspension to a 37°C water bath. The mixture was then sealed and shaken vigorously for a further 3 days at 37°C, followed by vacuum filtration and storage at 37°C. All solutions and solids were pre-warmed in an incubator to 37°C. Solid NaCl and Tris were added separately to the CaOx solution and the pH adjusted quickly (~1 min) with dilute HCl. This buffer was stored at 37°C prior to use.

Proteolysis and limited dissolution

A total of 0.25 mg samples of each of the crystal types listed in Table 1 were incubated at 37°C for 16 h with agitation (100 rpm) in 0.5 ml of a solution of 12.5 mmol/l Tris, pH 6.0, to which solid CaOx had been added, and 2 units/ml of either cathepsin D, thrombin or Proteinase K to produce a solution unsaturated with respect to CaOx. FESEM observation confirmed that limited crystal dissolution occurred under these conditions.

Limited dissolution

A total of 0.25 mg samples of each of the crystal types listed in Table 1 were incubated at 37°C for 16 h with agitation (100 rpm) in a 0.5 ml solution of the unsaturated CaOx solution described above.

UF crystals

In all cases, crystals deposited in ultrafiltered urine (WUF and BUF) served as negative controls. These crystals were then treated in one of three ways. UF crystals were either retained as controls, incubated in the saturated CaOx solution, or treated with 2 units/ml cathepsin D in the saturated CaOx solution.

Preparation of crystals for FESEM

After 16 h, aliquots (0.2 ml) were removed, suspended in distilled water (2 ml) and filtered (0.22 μm). The filters were dried, glued onto aluminium stubs and coated with Au/C using a Denton DV-502 high vacuum evaporator (Denton Vacuum, Cherry Hill, N.J., USA). The crystals were examined using a field emission scanning electron microscope (Philips XL30) at an operating voltage of 10 kV.

Results

Western blotting

Western blotting (Fig. 1) identified a large proportion of the protein from WF and BF as UPTF1 and OPN. It is evident that BF included considerably more UPTF1 and OPN per mg of CaOx than WF, although this could not be quantified. Multiple bands were observed in the proteins from the crystals from both population groups blotted for OPN (Fig. 1b), which is in agreement with a previous report that the molecular weight of OPN varies widely from 20–75 kDa in SDS-PAGE systems [25]. The multiple bands observed in the present study would have resulted from degradation of OPN by urinary proteases [14], since we have shown in other studies (data not presented) that the OPN pattern alters markedly when

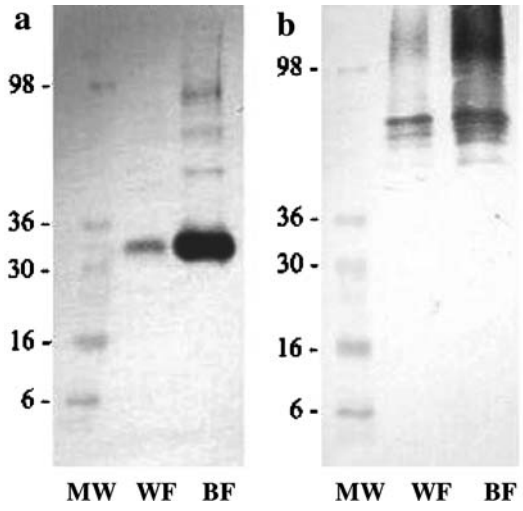

Fig. 1 Western blot of the WF and BF CaOx urinary crystals immunoblotted for **a** prothrombin and **b** osteopontin. *MW*, prestained molecular weight marker; *WF*, crystals from filtered urine of white subjects; *BF*, crystals from filtered urine of black subjects

urine is collected in the presence of the preservative thimerosal, as has been shown by Hoyer et al. [26].

FESEM data

Crystals deposited from ultrafiltered urine

Electron microscopy showed that the effects of various treatments were qualitatively similar for the WUF and BUF crystals. Thus, only representative micrographs of UF crystals are shown in Fig. 2. In the control crystals, a few COMs had surface detail, particularly on the {101} face (Fig. 2a), which strongly suggested that removal of some low molecular weight (< 10 kDa) intracrystalline components may have occurred, although most were smooth (Fig. 2b). The COD crystals had little surface texture (Fig. 2b) and appeared to be solid, as indicated by the interior structure of a fractured crystal (Fig. 2c). Crystal shape and texture of COM (Fig. 2d) and COD crystals (Fig. 2e) were largely unaffected by incubation in the saturated CaOx solution. Both COM (Fig. 2f) and COD crystals (Fig. 2g, h) were unaffected by digestion with cathepsin D.

The ultrastructure of UF crystals both before and after protease treatment was similar to those contained in a previous report [11]. The lack of internal ultrastructure observed was consistent with the absence of protein (> 10 kDa) observed on SDS-PAGE (data not shown) and justified the use of the UF crystals as negative controls.

Crystals deposited from filtered urine

The images of crystals obtained from filtered urine were not notably different from those deposited from ultrafiltered urine dosed with either WF1 or BF1. Thus, only representative micrographs of COM and COD crystals before treatment are presented in Fig. 3. A field view of WF (Fig. 3a) shows the predominance of bipyramidal COD crystals from the white subjects, although some small oval-shaped COM crystals were also observed. A similar view of BF (Fig. 3b) taken at the same magnification shows the predominance of COM crystals from the black subjects. Aside from this distinction in crystal morphology, no other differences between the respective samples from the two population groups were observed.

Several COM crystals showed surface detail on the {010} face (Fig. 3c). Fine fissures were observed on the {101} face (Fig. 3d) as well as in the interior of COM crystals (Fig. 3e), which were consistent with the binding and inclusion of urinary proteins during growth. The core of COM crystals appeared to be more porous than near the periphery (Fig. 3e). In contrast, most COD crystals showed little evidence of erosion, being relatively smooth and solid (Fig. 3f).

Proteolysis study

Figure 4 shows CaOx crystals precipitated from filtered urine, which were incubated in a saturated CaOx solution or in the same solution containing three different proteases. In the absence of proteases, most COM crystals remained intact (Fig. 4a), while some COD crystals developed slightly jagged edges and erosion on the {101} faces (Fig. 4b). Incubation with cathepsin D resulted in varying degrees of proteolysis of COM crystals. In the most severe cases, complete digestion of the crystal core occurred, leaving a doughnut shaped exterior shell (Fig. 4c), which confirmed that protein was most abundant at the crystal centre. COD crystals appeared relatively intact after treatment with cathepsin D (Fig. 4d). Fissures were evident on COM crystals (Fig. 4e treated with thrombin), but COD crystals (Figure 4f) were not notably affected. However, some crystal fragments caused by fracturing were observed on both COM and COD crystal surfaces (Fig. 4f) after incubation with thrombin. This may indicate that the crystals were coated with excess thrombin, which caused adhesion of the fragments to the crystal surfaces.

After incubation with Proteinase K, some COM crystals underwent surface erosion (Fig. 4g). Considerable surface etching of COD crystals was also observed with this treatment (Fig. 4h). The {101} faces of these COD crystals were more affected than those that had been incubated in the saturated CaOx solution alone (Fig. 4b).

Proteolysis and dissolution study

Electron micrographs of CaOx crystals incubated in the unsaturated CaOx solution in the presence of the three proteases are shown in Figs.5, 6, 7.

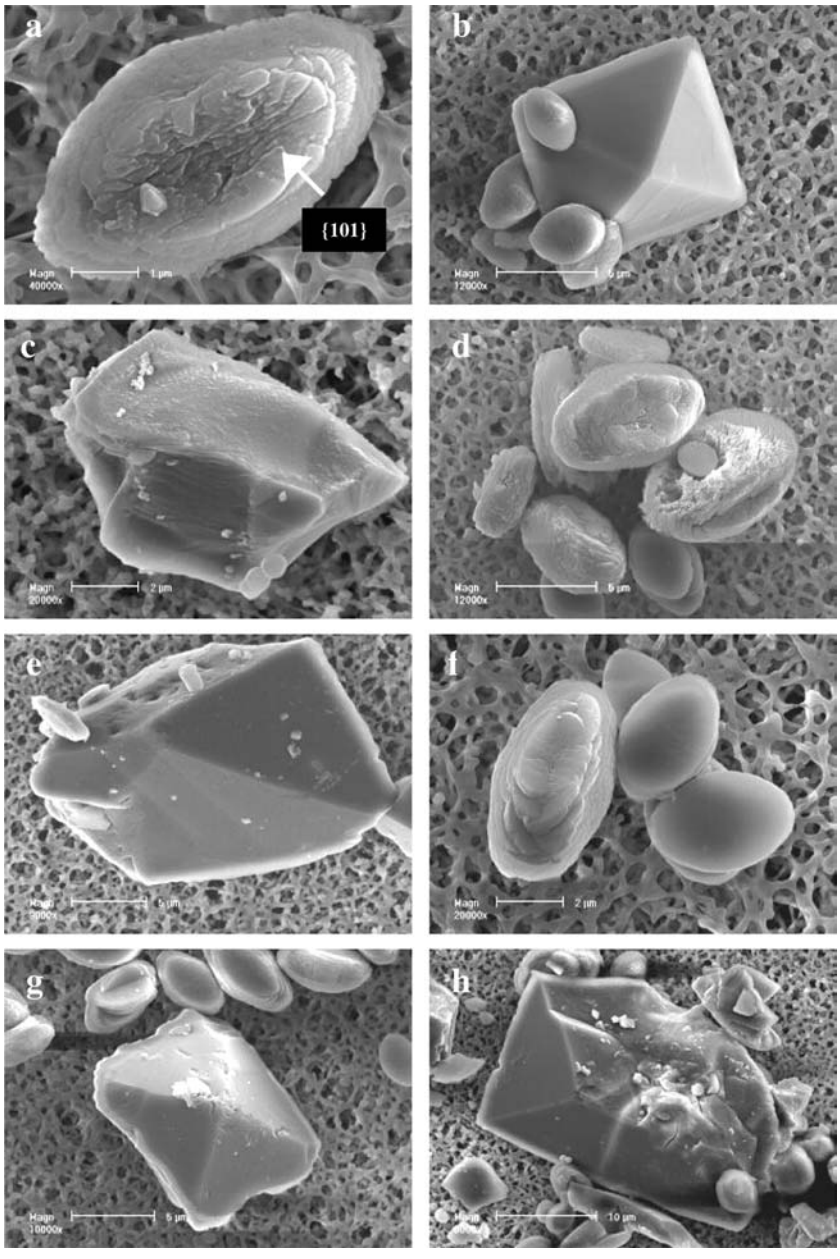

Fig. 2 FESEM images of fractured CaOx crystals from ultrafiltered urine. The following crystals and treatments are depicted: **a** COM and **b**, **c** COD crystals washed with water; **d** COM and **e** COD crystals incubated in a buffer saturated with CaOx; **f** COM and **g**, **h** COD crystals treated with cathepsin D in a buffer saturated with CaOx

Treatment with cathepsin D in an unsaturated CaOx solution

In the presence of cathepsin D, COM crystals were eroded and degraded to varying degrees. In some crystals, only surface detail on the {101} face was evident (Fig. 5a), while in others, disintegration was more significant and subcrystallites were observed (Fig. 5b). The effect of cathepsin D appeared to be most advanced in the COM crystal depicted in Fig. 5c, in which numerous closely packed subcrystalline particles were evident and most of the crystal core appeared to have been excavated by the treatment. The majority of COD crystals were unaffected and appeared to be relatively smooth and solid after treatment (Fig. 5d). However, some surface pitting and deformation of COD crystals were observed (Fig. 5e), as well as slightly eroded edges (Fig. 5f). There was no apparent dissolution when cathepsin D was used in the unsaturated CaOx solution.

Treatment with thrombin in an unsaturated CaOx solution

After treatment with thrombin, the interiors of the COM crystals (Fig.6a, b) were similar to those before treatment (compare Fig. 3e), although some porosity was evident. Crystal fragments caused by fracturing were frequently observed on the surface of COM (Fig. 6b) and COD crystals after incubation with thrombin. As suggested above, this may indicate that the crystals were coated with excess thrombin, which caused adhesion of the fragments to the crystal surfaces, and also that the

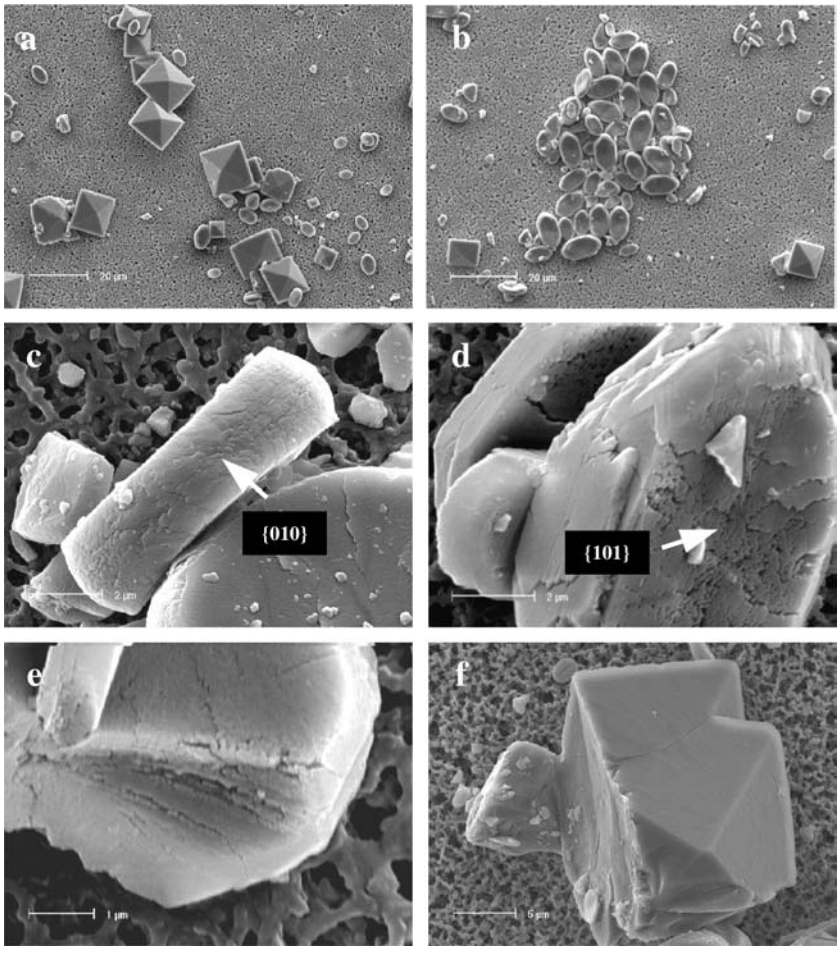

Fig. 3 FESEM images of fractured CaOx crystals precipitated from filtered urine before treatment. **a** Field view of WF, **b** field view of BF, **c**–**e** COM, **f** COD

degree of undersaturation of the CaOx solution was insufficient to cause dissolution of the fragments. The presence of a surface sheen upon some COD crystals (Fig. 6c) supports the notion of a surface thrombin coating, which also masks what appeared to be significant surface etching (Figure 6d).

Treatment with Proteinase K in an unsaturated CaOx solution

Although some were largely unaffected (e.g., the large COM in Fig. 7a), most crystals incubated with Proteinase K in the unsaturated CaOx solution showed evidence of erosion and considerable dissolution. Many COM crystals, which were deformed (Fig. 7b), had apparently dissolved from the interior, since they were concave in appearance (Figure 7c). In the latter case, a large amount of organic material was evident on the filter papers (e.g., around the small COM in Fig. 7a) and fewer crystals were observed than in the corresponding control samples. Both observations are consistent with the occurrence of significant dissolution. The COD crystals appeared to be relatively solid inside (Fig. 7d), but several had jagged edges (Fig. 7d, e) that appeared to consist of smaller crystallites with the same morphology as the parent crystal. Many CODs (Fig. 7f), but not all, showed evidence of significant erosion on the crystal surfaces.

Dissolution study

As occurred with all treatments, effects on individual crystals varied, with some crystals incubated in the unsaturated CaOx solution alone showing evidence of considerable etching and deformation and others remaining largely unaffected. Crystals from different batches, and from blacks and whites, were qualitatively similar, so no distinction is drawn between them. However, the effects of dissolution on COM and COD crystals were considerably different; therefore, the findings are depicted separately.

Dissolution of COM crystals

COM crystals that had been incubated in the unsaturated CaOx solution alone, at presumed, successive stages of dissolution, are presented in Fig. 8. The arrows indicate the proposed pathway of dissolution from a single, intact COM crystal (Fig. 8a) to what

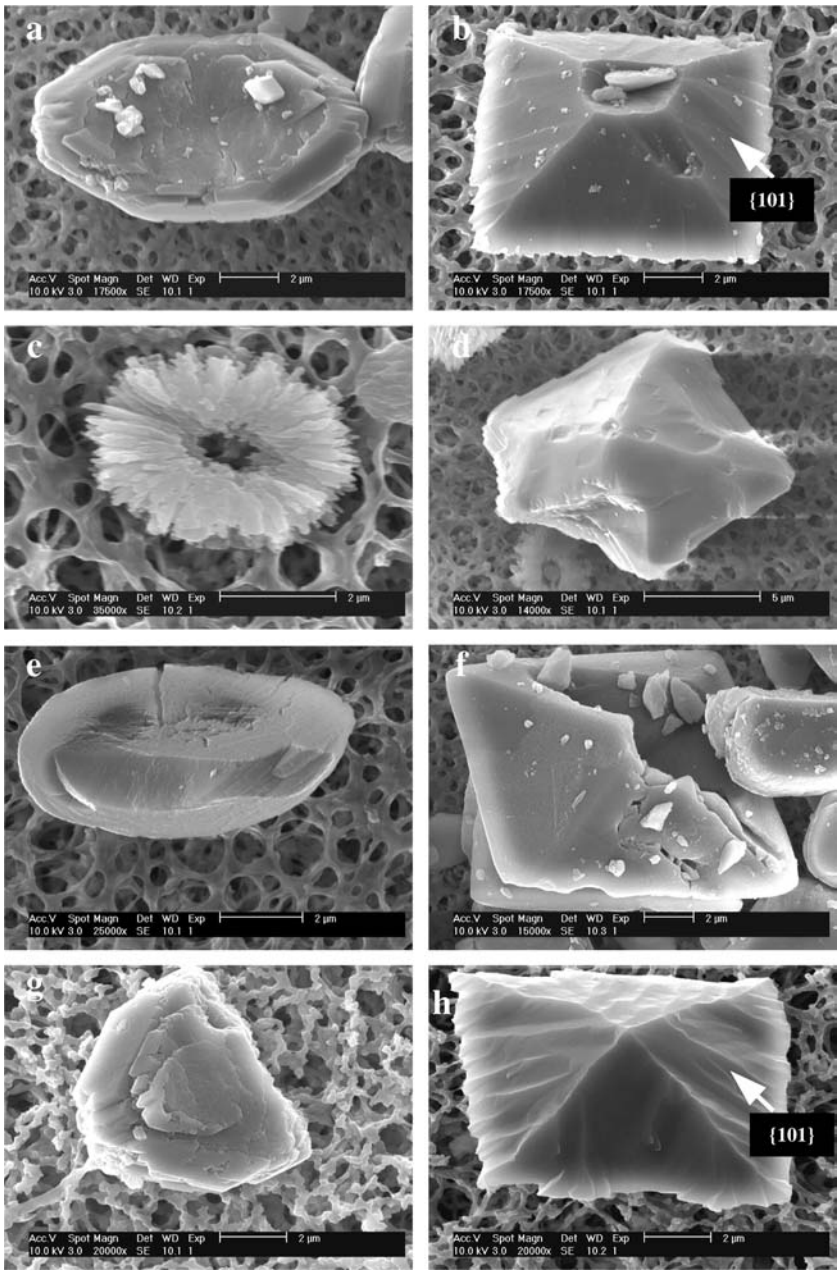

Fig. 4 FESEM images of fractured COM and COD crystals from filtered urine treated with three proteases in a saturated CaOx buffer. **a** COM and **b** COD crystal incubated in a saturated CaOx buffer alone; **c** COM and **d** COD crystal treated with cathepsin D in buffer saturated with CaOx; **e** COM and **f** COD crystal treated with thrombin in a buffer saturated with CaOx; **g** COM and **h** COD crystal treated with Proteinase K in a buffer saturated with CaOx

appeared to be its smallest component fragments (Fig. 8f). Apparently whole, single COM crystals first showed evidence of dissolution from the interior, seemingly consisting of concave structures covered with an organic skin (Fig. 8b). This skin, which is also visible in Fig. 8c, became more evident as dissolution progressed. More advanced dissolution coincided with the deposition of increasing amounts of organic material on the filter surface (Fig. 8d, e). Tiny, amorphous crystallites, which appeared to comprise amorphous particles consisting of mineral embedded in organic matter, were observed in crystals that were probably in the final stage of dissolution (Fig. 8f). Such sub-crystalline particles have been described previously [10, 11].

Dissolution of COD crystals

Figure 9 shows a similar series of micrographs depicting COD crystals at presumed, successive stages of dissolution. The arrows indicate the proposed pathway of dissolution from a single, intact COD crystal (Fig. 9a) to, perhaps, the exposure of its component crystallites (Fig. 9d). Apparently single, whole CODs fragmented progressively, first at their edges (Fig. 9b) and then on the {101} faces (Fig. 9c). Fracturing characteristically occurred along a common plane, namely the middle of the {101} face (as depicted in Fig. 9c), and revealed tiny and geometrically arranged bipyramidal subunits (Fig. 9d), which undoubtedly represent crystallites. It is evident that the dissolution of COD crystals is quite

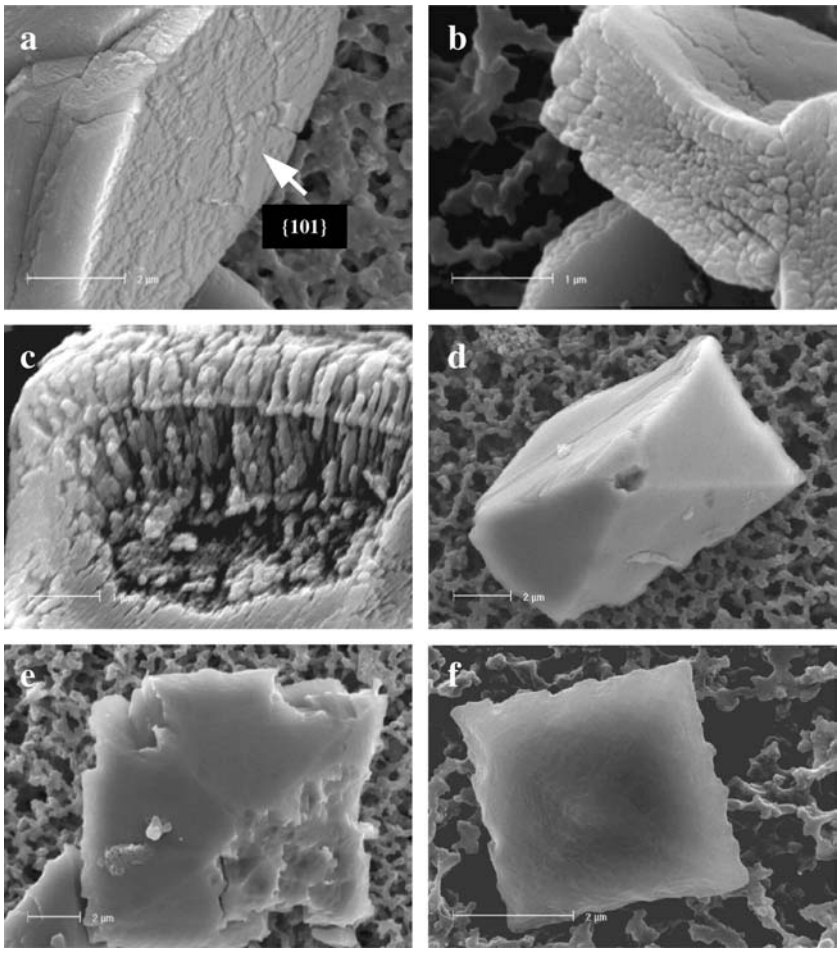

Fig. 5 FESEM images of fractured **a**–**c** COM and **d**–**f** COD crystals treated with cathepsin D in a buffer undersaturated with CaOx. The crystals were precipitated from the following urines: **a** F, **b** F, **c** UF + BF1, **d** F, **e** F and **f** UF + WF1

different from the COM crystals and, moreover, that there does not appear to be a collection of organic material at the crystal core. Nor is there evidence of significant organic material on the surrounding filter surfaces.

Discussion

The results presented here have demonstrated that CaOx crystals precipitated from filtered urine samples of both white and black subjects are eroded by treatment with proteases, which caused etching, degradation and, in some instances, excavation of the crystals to varying degrees. These changes could not have resulted from mineral dissolution, since proteolysis was carried out in a solution saturated with CaOx. The fact that crystals deposited from ultrafiltered urine were relatively solid and had smooth surfaces confirmed that the observed erosion must have resulted from the removal of intracrystalline proteins. These findings are in agreement with previous reports by Ryall and co-workers [10, 11].

In the most advanced cases of degradation, considerable excavation of the crystal interiors was observed, exposing an array of tightly packed, subcrystalline particles. The removal of the core of some, but not all, COM crystals by protease treatment suggests, as has been previously reported [11], that the protein was concentrated in the central region of the crystal and not near the edges. This is consistent with the condensation of protein and the subsequent precipitation of mineral around it. As the urine is depleted of protein, the proportion of surrounding mineral shell increases [13].

Partial dissolution studies of the crystals permitted the observation of the different ultrastructures of COM and COD crystals, as well as stages of their fragmentation. Whole, disc-shaped COM crystals first showed signs of dissolution at the centres of their {101} faces, which is consistent with the location of protein at the core of the crystal structure, as previously described [11]. In the latter stages of dissolution, tiny COM crystallites were observed amongst the organic material that had leached onto the filter paper. These particles, which may be amorphous composites of organic and inorganic material, were previously reported by Ryall and co-workers in COM crystals [10], and resemble similar amorphous subunits seen by Addadi and co-workers in a study of the mineralisation of the marine sponge [27].

Disintegration of the bipyramidal COD crystals first occurred at their periphery, where jagged edges were

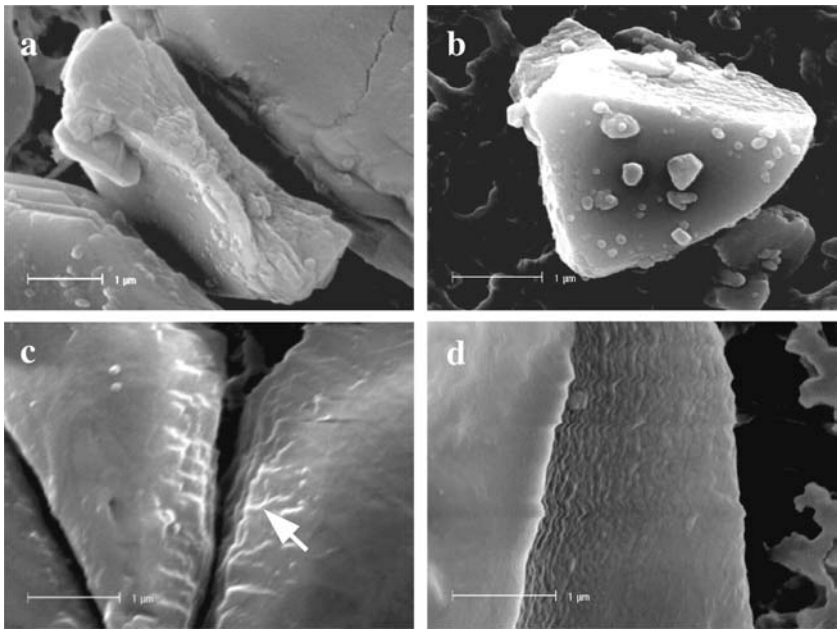

Fig. 6 FESEM images of fractured **a**–**b** COM and **c**–**d** COD crystals treated with thrombin in a buffer undersaturated with CaOx. All of the crystals were precipitated from filtered urine

evident, apparently representing outlined margins of subcrystalline particles. Resembling the morphology of the larger structure, these are likely to be crystallites of which the apparently single parent COD crystals are composed. Distinct fracture planes were also observed, typically in the centre of the {101} faces. The strengthening of crystal planes by proteins against fracturing has been described previously in the sea urchin spine [27],

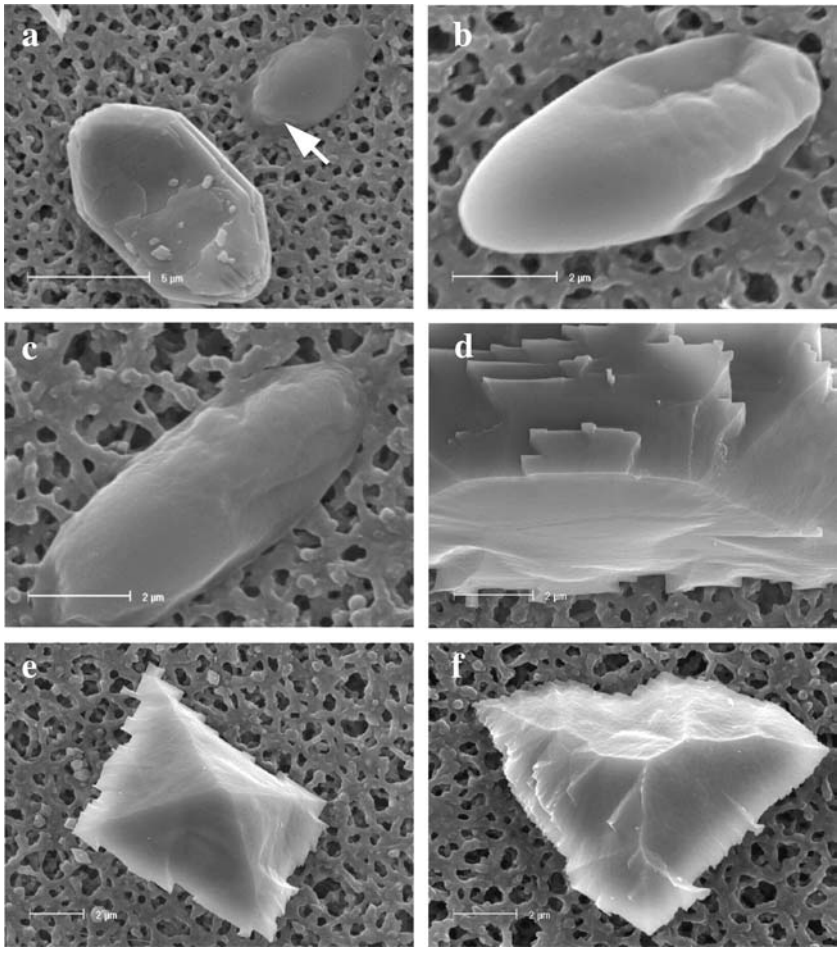

Fig. 7 FESEM images of fractured **a**–**c** COM and **d**–**f** COD crystals treated with Proteinase K in a buffer undersaturated with CaOx. The crystals were precipitated from the following urines: **a** F, **b** UF + BF1, **c** F, **d** F, **e** F and **f** UF + WF1

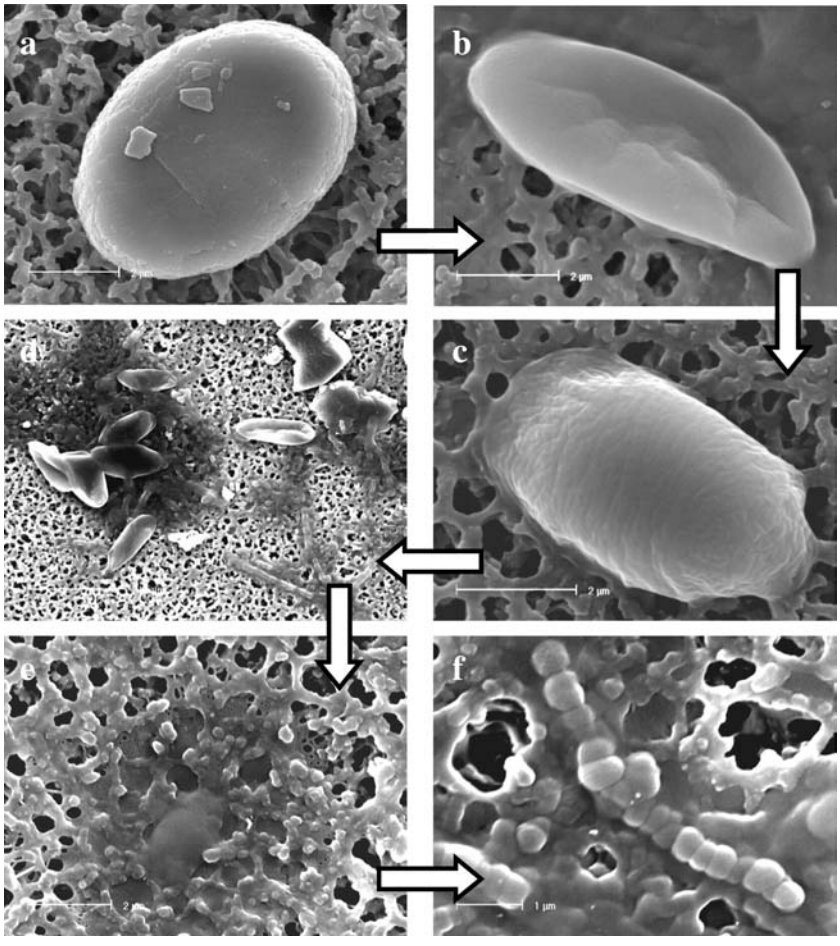

Fig. 8 FESEM images showing COM crystals at presumed, successive stages of dissolution in a buffer undersaturated with CaOx. **a** Depicts a typical COM crystal before treatment

and would seem to be a plausible explanation for the fracture planes observed here in urinary crystals that had been partly shattered prior to proteolysis.

Observation of simultaneous dissolution and proteolysis was achieved by the use of a buffer undersaturated with CaOx in conjunction with protease treatment. Since

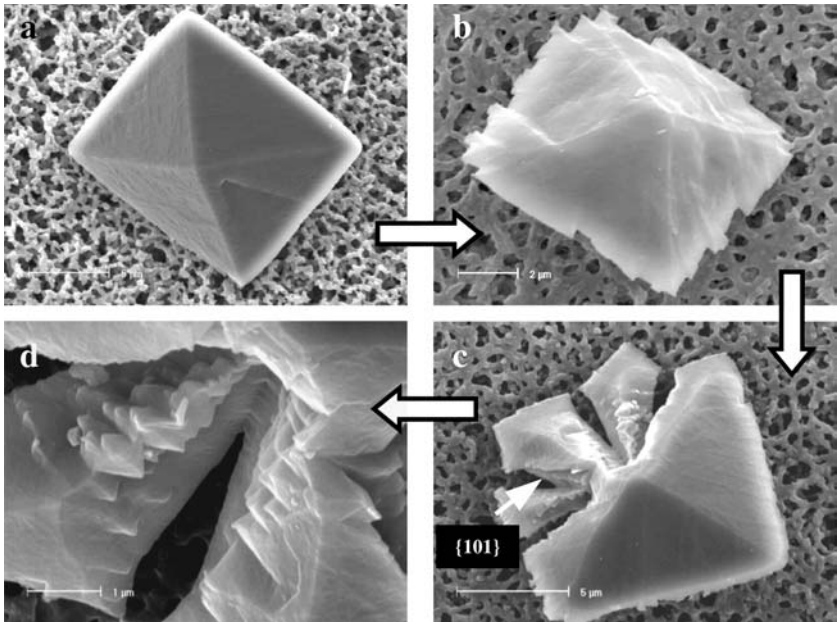

Fig. 9 FESEM images showing COD crystals at presumed, successive stages of dissolution in a buffer undersaturated with CaOx. **a** Depicts a typical COD crystal before treatment

these two processes are likely to occur concurrently in vivo, these observations are possibly the most physiologically relevant, and it was therefore not surprising that this caused more significant erosion and degradation of the crystals than occurred in those subjected to either one of the processes alone. Of the three proteases tested, cathepsin D caused the most significant crystal erosion, and also caused noticeably more degradation of COM crystals than COD crystals, which may reflect selectivity by cathepsin D for proteins associated with the COM mineral, of which UPTF1 is predominant [14, 15]. Thus further proteolysis studies of this protein should include treatment with cathepsin D. The results obtained in the present study with cathepsin D are of particular interest since the protease is found in cell lysosomes and is active throughout the tubules of the human nephron [29]. Thus, it is possible that cathepsin D could mediate the dismantling and dissolution of CaOx crystals if they are internalised by renal cells in vivo.

The effects of both thrombin and Proteinase K on COD crystals were noteworthy. Although thrombin appeared to coat the surface of some CODs, it also caused etching of their surfaces. The apparent selectivity of thrombin for COD crystals is consistent with the susceptibility of OPN to endogenous proteolysis in urine [28] and its greater abundance in COD crystals than in COM [14, 15]. Considerable dissolution was observed along with surface erosion of COD crystals after incubation with Proteinase K. It is possible that more extensive proteolysis by Proteinase K resulted in greater dissolution. Furthermore, the surface erosion of COD crystals (but not COM) suggests selectivity of Proteinase K for proteins associated with COD, although the reason for this is not known.

No discernible differences in the relative response of crystals from white and black subjects to the treatments were noted. However, all COM crystals (whether from white or black subjects) appeared to be more susceptible to degradation and dissolution than COD crystals, which probably reflects their unique crystal structures. Other differences have been previously reported between the two CaOx hydrates. These include a higher surface charge of COM crystals [30] and greater surface absorption of polyphosphate by COMs compared with COD [31]. The distinctive occurrences of the CaOx hydrates in the two population groups, as well as their relative response to proteolytic treatment, are thus important and warrant further analysis.

A study by Riese and co-workers was the first to report the binding of COM crystals to cultured renal cell surfaces [32], an observation which others have confirmed using several renal cell lines [e.g. 5, 33]. Crystal adherence to the renal epithelium is now widely regarded as a probable mechanism of stone formation, in addition to blockage of the nephron caused by crystal aggregation [2]. Crystal adhesion appears to be under stereospecific control since 50% more COM than COD crystals bind to inner medullary collecting duct cells from rats [34]. However, although crystal adhesion may at first appear to promote stone formation by providing a site for further crystal deposition and encouraging tubular blockage, it is also possible that the attached crystals will be phagocytosed, thus facilitating their degradation by intracellular proteases. Phagocytosis has been observed in cultured monkey cells treated with exogenous COM [35] and COD crystals [1], which were internalised and dissolved over a period of weeks within the cell lysosomes [2]. In fact, it has been proposed that crystal attachment and subsequent intracellular destruction may be an unexpected means by which to avert stone formation [2, 3], a notion that is supported by reports of raised levels of lysosomal [28, 36] and brush border membrane [37] proteases in the urine of stone formers. Furthermore, cultured renal cells challenged with CaOx crystals show elevated lysosomal protease activity [38], which also suggests that attachment of crystals and their subsequent intracellular destruction may occur in vivo.

It has been proposed that the selective formation of COD, rather than COM crystals, protects against urolithiasis, since they would be less likely to adhere to renal tubular cells [39]. However, the results of the present study suggest that internalised COM crystals will be more readily destroyed than COD. The predominance of COM amongst the black population could thus prove to be a decisive factor in their relative protection from stone disease. While it is tempting to speculate that the lower stone rate amongst blacks can be attributed to their efficient destruction of retained COM crystals, further investigations comparing the attachment and dissolution of urinary crystals from white and black subjects using a renal cell culture model are clearly required.

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