Published in final edited form as:

BJU Int. 2010 March ; 105(5): 708–715. doi:10.1111/j.1464-410X.2009.08816.x.

The effects of intracrystalline and surface-bound proteins on the attachment of calcium oxalate monohydrate crystals to renal cells in undiluted human urine

Phulwinder K. Grover, Lauren A. Thurgood, Tingting Wang, and Rosemary L. Ryall
Urology Unit, Department of Surgery, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, Australia

Abstract

Objective—To compare the binding to Madin-Darby canine kidney (MDCK)-II cells of: (i) inorganic calcium oxalate monohydrate (iCOM) crystals and COM crystals precipitated from urine containing different concentrations of protein; and (ii) urinary COM crystals containing intracrystalline and intracrystalline + surface-bound protein.

Materials and methods—Urinary COM crystals were generated in sieved (sCOM), centrifuged and filtered (cfCOM), and ultrafiltered (ufCOM) portions of a pooled human urine and their adhesion to MDCK-II cells was compared using six different ultrafiltered urine samples as the binding medium. Crystal matrix extract (CME) was prepared by demineralizing calcium oxalate crystals precipitated from human urine and used to prepare COM crystals with intracrystalline, and intracrystalline + surface-bound CME at protein concentrations of 0, 0.05, 0.1, 0.5 and 5.0 mg/L. The amount of protein associated with the crystals was qualitatively assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting, using prothrombin fragment 1 (PTF1) as a marker. Protein concentration was determined in sieved, centrifuged and filtered, and ultrafiltered fractions of 10 additional urine samples.

Results—The median crystal attachment in the six urine types decreased in the order iCOM > ufCOM > cfCOM = sCOM, in inverse proportion to the concentration of protein in the solution or urine from which they were precipitated. sCOM and cfCOM crystals bound $\approx$ 23% less than iCOM crystals. The attachment of COM crystals generated in the presence of increasing concentrations of CME proteins was unaffected up to a concentration of 5 mg/L, but binding of crystals containing the same concentrations of intracrystalline + surface-bound proteins decreased proportionally at protein concentrations from 0 to 5.0 mg/L.

Conclusion—Inorganic COM crystals bind significantly more strongly to MDCK-II cells than urinary crystals precipitated from sieved, centrifuged and filtered, and ultrafiltered urine, and binding affinity is inversely related to the concentration of protein in the urine in which they are formed. While both intracrystalline and superficial CME proteins reduce the attachment of COM crystals to MDCK-II cells, those located on the crystal surface have a greater influence than those incarcerated within the mineral bulk. Future cell–crystal interaction studies should use urinary crystals and be performed in human urine.

Keywords

calcium oxalate; urine; calculogenesis; nephrolithiasis; urinary calculi

Correspondence: Phulwinder K. Grover, Department of Surgery, Flinders Medical Centre, Bedford Park, SA 5042, Australia. pk.grover@flinders.edu.au.

Conflict of interest The authors have no conflict of interest

Introduction

In the investigation of urolithiasis, cell-culture models have been invaluable for addressing numerous aspects of crystal–cell interactions that would be impossible to explore using animal models. Using such models, it has been shown that phosphatidylserine [1], proteins containing sialic acid [2,3], collagen IV [4], an acidic fragment of nucleolin-related protein [5], Annexin-II [6], osteopontin [7,8] and hyaluronan [9] mediate the attachment of calcium oxalate (CaOx) monohydrate (COM) crystals to renal epithelial cells. On the other hand, fibronectin [10], osteopontin [11], bikunin [12], heparan sulphate/syndecan-1 [13], hepatocyte growth factor [14], Tamm-Horsfall glycoprotein (THG) [11] chondroitin sulphates A and B [11], heparan sulphate [11], TGF β_2 [15] and undifferentiated urinary macromolecules [16–18] have been reported to reduce the adhesion of COM crystals. Inhibitory effects of macromolecules have been shown by altering the macromolecular composition of either the incubation medium in which crystal attachment was assessed [10,11,12,15,19] or the surfaces of the renal tubular cells [3,5,6,8,20,21]. However, the physiological relevance of those studies to renal stone formation is limited because in all instances they used only inorganic COM (iCOM) crystals. These differ in size, morphology and physicochemical properties from urinary COM crystals, which are also invariably and irreversibly associated with selected urinary proteins, both upon their surfaces and incarcerated within the mineral bulk [22,23]. While it is intuitively obvious that superficially bound proteins would be likely to affect the binding of crystals to cells, the possible influence of intracrystalline proteins is less evident. To date, the effects of intracrystalline proteins on crystal–cell adhesion have not been specifically examined.

An additional, crucial factor in all previous studies examining the effect of macromolecules on crystal adhesion to renal cells has been the use of Tris-HCl [12], PBS [6,11,15,19,24] or other aqueous inorganic solutions [3,5,8,20,21] as the binding medium, rather than human urine, which is the medium in which crystals would attach to the urothelium *in vivo*. As the composition of urine is far more complex than any aqueous solution, and it has already been documented to vary widely both daily and seasonally, even from the same individual, no synthetic solution can adequately replace real urine. This view is bolstered by a recent report which showed that the attachment of iCOM crystals to Madin-Darby canine kidney (MDCK)-II cells in an aqueous medium differs from that in urine [18], with binding being significantly affected by both the macromolecular and low molecular weight components content of the urine [18].

In the present study our aims were to compare quantitatively, using undiluted human urine as the binding medium, the attachment to MDCK-II cells of iCOM and urinary COM crystals containing different amounts of proteins. In addition, we compared the binding of urinary COM crystals containing only intracrystalline proteins with that of the same crystals which also had urinary proteins bound to their surfaces.

Materials and methods

[^{14}C]-labelled iCOM crystals were generated as described previously [25]; 24-h urine specimens collected from five healthy laboratory colleagues were pooled and divided into three equal portions. The first portion was strained through a 70- μm sieve, the second was centrifuged at 10 000 g and then filtered (CF) through a 0.22- μm filter (GVWP14250, Millipore Corporation, Billerica, MA, USA), and the third was ultrafiltered (UF) through a regenerated cellulose cartridge (cat # CDUF001LC, Millipore Corporation) with a nominal relative Mr threshold of 10 kDa, as described earlier [18]. [^{14}C]-labelled COM crystals were prepared from the sieved (sCOM), CF (cfCOM) and UF (ufCOM) urine samples as detailed previously [25]. To ensure the complete removal of any proteins that were not irreversibly

bound to their surfaces, the crystals were suspended in a volume of distilled water equivalent to a tenth that of the urine used to grow them, vigorously vortex-mixed and centrifuged at 2000 g for 10 min at 20 °C. This washing cycle was repeated nine times, after which the crystals were lyophilized and stored at – 20 °C for later use.

To prepare CaOx crystal matrix extract, 24-h urine samples collected from eight healthy laboratory colleagues were pooled and centrifuged and filtered, as above. CaOx crystals were precipitated in the sample and demineralized to yield crystal matrix extract (CME) as described previously [23]. A portion of the lyophilized CME was analysed by SDS-PAGE and Western blotting, and the remainder was stored at – 70 °C for later use.

To prepare [¹⁴C]-labelled COM crystals with intracrystalline and intracrystalline + surface-bound CME proteins, 24-h urine samples collected from six healthy laboratory colleagues were pooled, CF and then UF, as above. The sample was divided, one half being used to prepare crystals containing increasing amounts of intracrystalline CME proteins, and the other to generate crystals with increasing quantities of intracrystalline + surface-bound CME proteins. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA), using human serum albumin (Sigma, St Louis, MO, USA) as a standard.

For COM crystals containing intracrystalline CME proteins, the UF sample was further divided into five aliquots to which a solution of CME was added to give final protein concentrations of 0, 0.05, 0.1, 0.5 and 5.0 mg/L. Radiolabelled COM crystals were deposited from these as described earlier [26]. The crystals were then filtered, washed thoroughly to remove any unbound proteins, lyophilized and stored as described previously [25].

For COM crystals containing intracrystalline + surface – bound CME proteins, the UF sample was treated identically to the first half as described above, except that 1 h after adding oxalate load, the same amount of the CME solution was added to each separate flask and the crystal suspension was incubated with shaking at 37 °C for a further 1 h. The final crystals therefore contained intracrystalline CME, as well as CME bound to their surfaces (intracrystalline + surface-bound CME). The crystals were then filtered, washed thoroughly, lyophilized and stored as described previously [25].

MDCK-II cells, which are a specific subtype of MDCK cells representative of proximal/distal renal epithelium, were generously provided by the late Dr Carl Verkoelen (Erasmus University, Rotterdam, the Netherlands). These cells were selected because they bind and internalize COM crystals, and have been used extensively in studies involving crystal–cell interactions, e.g. [27]. They were grown using conditions described by Kumar *et al.* [16]. High-density quiescent cultures prepared in 35-mm plastic plates [11] were used for attachment experiments.

Crystal binding experiments

To avoid interference from endogenous macromolecules, the attachment of sCOM, cfCOM, ufCOM and iCOM crystals to MDCK cells was done in individual UF urines which were collected from healthy laboratory colleagues and processed as described above.

Preliminary experiments showed that all crystals bound maximally by 10 min; thus crystals were left in contact with the cells for 15 min before removal of unattached crystals and determination of bound radioactivity. Crystal attachment experiments were conducted essentially as described previously [18]. Briefly, the culture medium was removed by aspiration and the cells were rinsed three times with 2 mL of the UF urine to be used as the binding medium; 2 mL of UF urine in which had been suspended 400 µg of [¹⁴C]-labelled,

sCOM, cfCOM, ufCOM or iCOM crystals was then added to each culture dish. After incubating the dishes for 15 min at 37 °C, the suspension was removed by aspiration and the cells were washed three times with the same UF urine. The attached crystals were dissolved by adding 2 mL of UF urine followed by 1 mL of concentrated HCl, and the cells were removed with a cell scraper. The suspension was clarified by centrifugation, and the supernatant (600 µL) was counted for 2 min in 3 mL of scintillation fluid (Ready Safe, Beckman Instruments, Palo Alto, CA USA) in a liquid scintillation counter (Beckman). The same sCOM, cfCOM, ufCOM and iCOM crystals were used for all experiments. Data were collected in triplicate and the experiment was repeated using five additional UF urine specimens as the binding medium. In all cases the pH of the UF urine was adjusted to 6.11.

The same method was used to determine the effect on attachment of crystals containing intracrystalline and intracrystalline + surface-bound CME, except that the experiment was performed using a new pooled UF urine collected from five healthy laboratory colleagues, prepared as described above.

All experiments included controls comprising cells incubated with iCOM crystals using PBS saturated with CaOx (pH 6.11) as the binding medium, to allow direct comparison of data among experiments [18]. Crystal attachment was calculated from the measured level of radioactivity associated with the cells, relative to that contained in the original crystal suspension. Values were then expressed as a percentage of the values in the control incubation containing PBS, which was taken to be 100% attachment.

The effect of urine processing on urinary protein concentration

A first morning urine sample was collected from 10 healthy individuals (four males, six females) with no previous history of stone formation. The absence of haematuria and nitrites from the urine was confirmed by dipstick (Combur® test strips, Roche Diagnostics, Germany). A portion of the urine was sieved (70 µm) and stored at 4 °C for later processing. The remaining urine was centrifuged for 20 min at 10 000 g (J2-21 M/E, Beckman Instruments), followed by pre-filtration (RW0314250; Millipore Corp.) and filtration (0.22 µm; GVWP14250; Millipore). A portion of this urine was stored at 4 °C for later use. The remainder was UF through a Millipore Prep/Scale Spiral Wound TFF module with a nominal threshold of 10 kDa (CDUF002LC) and then stored at 4 °C. The protein concentration in each sample was determined as described above.

Lyophilized samples were mixed with reducing sample buffer and separated by SDS-PAGE as described previously [18]. Gels were stained with silver. Western blotting was performed as described previously [23] using identical antibodies to those used in an earlier study [18].

The study was reviewed and approved by the Committee on Clinical Investigation (Ethics Committee) of the Flinders Medical Centre.

Each experiment was performed in triplicate and data were plotted as the mean (SEM); no error bars indicate that the error was smaller than the symbol used for the means. The data sets were compared statistically using Student's *t*-test, with *P* < 0.05 considered to indicate statistical significance.

Results

The CaOx crystals from which the CME was prepared consisted predominantly of COM mixed with a smaller proportion of CaOx dihydrate (COD). Because SDS-PAGE analysis showed protein bands identical to those we reported previously [23], the gels are not shown. A major band running at ≈31 kDa was shown by Western blotting to be prothrombin

fragment 1 (PTF1). Western blotting also confirmed the presence of osteopontin, migrating as a smear from 48–70 kDa, and human serum albumin, which ran as a single band at ≈ 50 kDa.

The mean protein concentrations of the 10 processed urine samples were: s, 47.3 (30.6) > cf, 24.8 (12.4) > uf, 2.2 (1.8) mg/L. The protein concentration in the inorganic crystals was assumed to be zero. Figure 1 shows the attachment of the sCOM, cfCOM, ufCOM and iCOM crystals to the cells using six different UF urine specimens as the binding medium. The median (range) attachment of sCOM (63%, 55–71%) and cfCOM (64%, 54–72%) crystals did not differ ($P > 0.05$), and both were significantly ($P < 0.01$) lower than that of the ufCOM (73%, 68–79%) crystals, which was significantly ($P < 0.01$) less than the value obtained with iCOM crystals (82%, 71–89%).

In Fig. 2 the median attachment values are plotted as a function of the mean concentration of protein remaining in the 10 urine samples after they were sieved, CF or UF; the control attachment value, obtained using iCOM crystals, is also included. Crystal adhesion correlated indirectly with the mean concentration of protein in the urine sample (or inorganic solution) from which the crystals were precipitated.

Figure 3a,b shows Western blots stained for PTF1 of the matrix extracts of the COM crystals containing intracrystalline CME, and intracrystalline + surface-bound CME, respectively. PTF1 was undetectable in crystals grown in UF urine containing no added CME, but appeared as a diffuse band at ≈ 31 kDa, the staining intensity of which increased relative to the final CME concentration. Although not quantitative, these data show that the amounts of intracrystalline PTF1, and consequently other CME proteins associated with CaOx crystals, are proportional to the concentration of CME in the urine from which the crystals are precipitated and in which they are incubated.

Figure 4 shows the degree of attachment of COM crystals associated with increasing amounts of intracrystalline CME proteins, or intracrystalline + surface-bound CME proteins. At CME protein concentrations up to 0.5 mg/L the attachment of crystals containing only intracrystalline CME was relatively constant, being $\approx 85\%$ of that of iCOM crystals in PBS, which was taken to be 100%. Adherence decreased slightly, but significantly to 80% ($P < 0.05$) at a CME concentration of 5 mg/L. However, adhesion of COM crystals precipitated in and coated with CME (intracrystalline + surface-bound CME) decreased in proportion to the final CME concentration. There was no real change between 0 mg/L and 0.05 mg/L, but attachment then decreased to 81.5% ($P < 0.05$), 73.5% ($P < 0.01$) and 65% ($P < 0.01$) at CME concentrations of 0.1, 0.5 and 5 mg/L, respectively.

Discussion

The binding of a cell to a substrate, such as a crystal surface, has been described as a complex, multistage process progressing from initial cell attachment, to spreading, to formation of focal adhesion [28]. While the first step might entail direct molecular association between the surfaces of the cell and substrate, the later stages require complex stereospecific interactions involving a retinue of cytoplasmic, transmembrane and extracellular proteins, which combine to create a stable attachment point [28,29]. Numerous factors influence interactions between crystals and cells, many of which affect the adhesive properties of the cell itself. These include membrane receptor molecules such as hyaluronan [30,31] and osteopontin [31,32], the chemical composition and pH of the ambient medium [33], cell polarity [34,35], cellular integrity [31,32,35], membrane fluidity [36], surface electrical charge [2] and phosphatidyl serine groups [1,37], cell type [38] and the proximity of the mineral surface, which can alter a protein's three-dimensional configuration [39]. The

ionic composition of the surrounding medium can also alter the conformation of proteins and thus, their binding potential; e.g. the binding of PTF1 to COM [23], osteopontin to COD [23,40] and matrix Gla protein to hydroxyapatite [41] are all profoundly affected by the ambient concentration of calcium. At the molecular level, the primary, secondary and tertiary structures of proteins are also known to influence their binding to crystal surfaces. The binding of PTF1 to CaOx depends on the presence of γ -carboxyglutamic acid residues near its N-terminus [42], while binding of osteopontin to hydroxyapatite relies on its component glutamic acid and aspartic acid residues [43], and degree of phosphorylation [44].

The nature of the underlying surface also has a profound effect on a cell's ability to interact with and adhere to it. Surface features that influence cell binding include its physical structure, charge, hydrophobicity/hydrophilicity, and chemistry [28], as well as alterations in topology at the nanometre scale [30]. It is not surprising therefore, that proteins, e.g. [10-12,14] and glycosaminoglycans [11,13] adsorbed to the surfaces of COM crystals can interfere with their ability to interact with and attach to renal epithelial cells. However, no previous studies have reported the effect on cell binding of proteins located *inside* crystals, despite the fact that COM crystals formed in human urine contain urinary proteins interred within their mineral phase [22,45-47]. Thus, the first aim of the present investigation was to compare the attachment of iCOM crystals to MDCK-II cells with that of COM crystals containing different concentrations of protein, using undiluted UF human urine as the binding medium.

Adhesion of the crystals decreased in the order iCOM > ufCOM > cfCOM = sCOM. Binding of sCOM crystals was $\approx 23\%$ less than that of the iCOM crystals, which is consistent with an earlier report showing that attachment of COM crystals precipitated from whole urine is reduced by a similar order of magnitude compared with crystals generated in inorganic, artificial urine [16]. Percentage binding values varied among the six different UF urine specimens by up to $\approx 20\%$. As the same crystals were used in all cases, and all macromolecules > 10 kDa had been removed from the urine samples, the variability must have been caused by low molecular mass components of the UF urine samples used as the binding media. Such molecules, which are known to influence crystal adhesion affinity [18], include unidentified proteins and peptides of < 10 kDa in mass, as well as low molecular weight species like citrate [11] and magnesium [48,49]. Nonetheless, crystal binding was also a function of the crystals themselves, as despite the variation in actual percentage attachment values among the six urine specimens, the decreasing trend, i.e. iCOM > ufCOM > cfCOM = sCOM, was consistent within each sample. Because the crystals had been exhaustively washed with sufficient distilled water to remove all proteins bound to their surfaces [47], that trend must have resulted from variations in the amount of intracrystalline proteins contained within them.

Centrifugation, filtration and ultrafiltration reduce the urinary protein concentration [18,50,51], which was quantitatively confirmed here using fresh urine specimens from 10 healthy individuals; the mean concentration of protein decreased in the order s (47.3 mg/L) > cf (24.8 mg/L) > uf (2.2 mg/L). PTF1 is the predominant protein in COM crystal matrix [52,53] and has been a useful qualitative marker to show that inclusion of proteins into crystals occurs in a dose-response manner [18,23]. This was confirmed in the present study, as the band density of PTF1 in Western blots of the demineralized COM extracts increased in tandem with the concentration of CME in the UF urine from which the test crystals were precipitated. It is reasonable to conclude therefore that the observed differences in binding between the sCOM, cfCOM and ufCOM crystals resulted from disparities in their intracrystalline protein content. This conclusion is supported by the observation that median percentage attachment of the crystals in the six ultrafiltered urine samples decreased

inversely with the mean concentration of protein present in the urine fractions from which they had been precipitated. The binding curve approached a plateau between the protein concentrations of the CF and sieved urine samples, for two possible reasons. First, centrifugation and filtration of urine removes most of the THG, which is the most abundant urinary protein and accounts for the high protein concentration in sieved urine [18,50,51]. However, it is not incorporated into COM urinary crystals [47]. Second, synchrotron X-ray diffraction studies have shown that while protein inclusion into COM is dose-dependent, the ability of the crystal to accommodate intracrystalline proteins is limited [47,54].

Given that binding is a surface-controlled phenomenon, it seems illogical that attachment of COM crystals to cells would be affected by the amount of protein contained within them. However, inclusion of proteins into crystals is known to disrupt their atomic array and create dislocations that alter the texture of the mineral phase [55]. If those effects were to extend to the surfaces of crystals, they could influence their ability to adhere to epithelial cells by altering, e.g. surface charge or topology. We have shown that proteins incarcerated within urinary COM crystals tend to be concentrated at their geometric centres [22,46,56]. Nonetheless, the physical distribution of intracrystalline proteins throughout the mineral bulk will depend on the concentration of protein in the medium in which they are formed. At low concentrations, proteins will be quickly depleted, allowing subsequent uninterrupted deposition of solute and producing a crystal in which the core contains protein, but the periphery of which consists principally of uninterrupted mineral [22,56]. However, at high concentrations, inclusion of protein will continue in parallel with deposition of fresh mineral, resulting in the formation of crystals with protein distributed throughout the structure and extending to the crystal surface [56]. Surfaces of crystals generated at low protein concentrations would therefore be more ordered and contain fewer perturbations than those precipitated at high concentrations, the surfaces of which would be covered with more numerous discontinuities and protruding 'tails' of proteins partly immersed in the mineral bulk. This effect, illustrated diagrammatically in Fig. 5, would explain the inverse relationship between crystal attachment and the concentration of protein in the medium from which they are precipitated, especially as the effects of adsorbed proteins might extend beyond their physically occupied volume [57]. It would also explain why CME did not affect crystal attachment except at a concentration of 5 mg/L (see below), at which point the crystal might have reached its full capacity to accommodate protein, some of which might not have been covered with intact mineral.

Crystals formed under physiological conditions contain both intracrystalline and surface-bound urinary proteins. Although we have shown that intracrystalline proteins affect the attachment of crystals to cells, intuitively at least, proteins attached to the crystal surface would be expected to have a more profound influence. Thus, the second aim of this investigation was to compare the binding of urinary COM crystals containing only intracrystalline proteins with that of the same crystals which also had urinary proteins bound to their surfaces. COM crystals were precipitated from samples of the same UF urine to which had been added CME at final concentrations from 0 to 5.0 mg/L. Those values were chosen because CME inhibits CaOx crystallization in undiluted urine over the same concentration range [58], and significant inclusion of CME proteins into COM crystals occurs at CME concentrations up to 1 mg/L [23].

Intracrystalline CME had no measurable effect on crystal binding up to a concentration of 1 mg/L and caused only a slight decrease at 1–5 mg/L. By contrast, the combination of both intracrystalline and surface-bound CME proteins caused a steady decrease in binding from 0.05 to 5.0 mg/L. However, alterations in percentage attachment were less than those observed when the bindings of iCOM, ufCOM, cfCOM and sCOM crystals in the six different urine samples were compared. In that experiment the difference in actual

percentage attachment between iCOM and ufCOM crystals was 9%, for a corresponding increase in protein concentration of only 2.2 mg/L, while in the CME experiment attachment decreased by only $\approx 5\%$ in response to an increase in CME concentration of 5 mg/L. In view of the large variability in the absolute percentage attachment values among the six individual urine samples, as discussed above, the discrepancy in binding values between the experiments can probably be attributed to differences between the concentrations of low molecular mass constituents in the urine samples used in the two experiments, or to differences in urinary concentrations.

In summary, this investigation showed that: (i) Urinary COM crystals bind with significantly less avidity to renal epithelial cells than do those consisting of pure inorganic mineral, and crystal attachment is less when urine (rather than PBS) is used as the binding medium. Therefore, future studies examining the effects of molecules on crystal adhesion to cultured cells should use urinary crystals and be performed in human urine. (ii) The binding affinity of COM crystals is inversely related to the concentration of protein in the urine in which they are formed. (iii) While both intracrystalline and superficial CME proteins reduce the attachment of COM crystals to MDCK-II cells, those located on the crystal surface have a greater influence than those incarcerated within the mineral bulk.

To date, the specific role of urinary proteins in the genesis of CaOx kidney stones remains unresolved. Nevertheless, they are known to inhibit CaOx crystal growth and aggregation [59,60], and the results presented here confirm that they inhibit the attachment of COM crystals to the renal epithelial cell membrane, irrespective of whether they are located inside or upon the surface of the mineral phase. Although intracrystalline proteins do not inhibit crystal adhesion as potently as those bound to the crystal surface, it is apparent that *in vivo* they might nonetheless fulfil dual roles in stone prevention as inhibitors of crystal growth, aggregation and attachment within the kidney, and if crystal retention and internalization do occur, as facilitators of intracellular crystal degradation and dissolution [26].

Acknowledgments

We gratefully acknowledge Grant No. NDDK 1 RO1 DK064050-01A1 from the National Institutes of Health, USA.

Abbreviations

CaOx	calcium oxalate
(i)CO(M)(D)	(inorganic) CaOx (monohydrate) (dihydrate)
s	sieved
UF/uf	ultrafiltered
CF/cf	centrifuged and filtered
THG	Tamm-Horsfall glycoprotein
MDCK	Madin-Darby canine kidney
CME	crystal matrix extract
PTF1	prothrombin fragment 1

References

1. Bigelow MW, Wiessner JH, Kleinman JG, Mandel NS. Surface exposure of phosphatidylserine increases calcium oxalate crystal attachment of IMCD cells. *Am J Physiol*. 1997; 272:F55–F62. [PubMed: 9039049]
2. Lieske JC, Leonard R, Swift H, Toback FG. Adhesion of calcium oxalate monohydrate crystals to anionic sites on the surface of renal epithelial cells. *Am J Physiol (Renal Fluid Electrolyte Physiol)*. 1996; 270:F192–F9.
3. Verkoelen CF, van der Boom BG, Kok DJ, Romijn JC. Sialic acid and crystal binding. *Kidney Int*. 2000; 57:1072–82. [PubMed: 10720959]
4. Kohri K, Kodama M, Ishikawa Y, et al. Immunofluorescent study on the interaction between collagen and calcium oxalate crystals in the renal tubules. *Eur Urol*. 1991; 19:249–52. [PubMed: 1855532]
5. Sorokina EA, Wesson JA, Kleinman JG. An acidic peptide sequence of nucleolin-related protein can mediate the attachment of calcium oxalate to renal tubule cells. *J Am Soc Nephrol*. 2004; 15:2057–65. [PubMed: 15284292]
6. Kumar V, Deganello FG, Lieske JC. Annexin II is present on renal epithelial cells and binds calcium oxalate monohydrate crystals. *J Am Soc Nephrol*. 2003; 14:289–97. [PubMed: 12538728]
7. Yamate T, Kohri K, Umekawa T, et al. Osteopontin antisense oligonucleotide inhibits adhesion of calcium oxalate crystals in Madin-Darby canine kidney cell. *J Urol*. 1998; 160:1506–12. [PubMed: 9751404]
8. Yasui T, Fujita K, Asai K, Kohri K. Osteopontin regulates adhesion of calcium oxalate crystals to renal epithelial cells. *Int J Urol*. 2002; 9:100–9. [PubMed: 12028300]
9. Verkoelen CF. Crystal retention in renal stone disease. A crucial role for the glycosaminoglycan hyaluronan? *J Am Soc Nephrol*. 2006; 17:1673–87. [PubMed: 16707562]
10. Tsujihata M, Miyake O, Yoshimura K, Kakimoto KI, Takahara S, Okuyama A. Fibronectin as a potent inhibitor of calcium oxalate urolithiasis. *J Urol*. 2000; 164:1718–23. [PubMed: 11025758]
11. Lieske JC, Leonard R, Toback FG. Adhesion of calcium oxalate monohydrate crystals to renal epithelial cells is inhibited by specific anions. *Am J Physiol (Renal Fluid Electrolyte Physiol)*. 1995; 268:F604–F12.
12. Ebisuno S, Nishihata M, Inagaki T, Umehara M, Kohjimoto Y. Bikunin prevents adhesion of calcium oxalate crystals to renal tubular cells in human urine. *J Am Soc Nephrol*. 1999; 10:S436–S40. [PubMed: 10541279]
13. Chikama S, Iida S, Inoue M, et al. Role of heparan sulphate proteoglycans (syndecan 1) on the renal epithelial cells during calcium oxalate monohydrate crystal attachment. *Kurume Med J*. 2002; 49:201–10. [PubMed: 12652971]
14. Tei N, Tsujihata M, Tsujikawa K, Yoshimura K, Nonomura N, Okuyama A. Hepatocyte growth factor has protective effects on crystal–cell interaction and crystal deposits. *Urology*. 2006; 67:864–9. [PubMed: 16600342]
15. Lieske JC, Toback GF. Regulation of renal epithelial cells endocytosis of calcium oxalate monohydrate crystals. *Am J Physiol (Renal Fluid Electrolyte Physiol)*. 1993; 264:F800–F7.
16. Kumar V, Farrell G, Lieske JC. Whole urinary proteins coat calcium oxalate monohydrate crystals to greatly decrease their adhesion to renal cells. *J Urol*. 2003; 170:221–5. [PubMed: 12796693]
17. Wiessner JH, Hung LY, Mandel NS. Crystal attachment to injured renal collecting duct cells. influence of urine proteins and pH. *Kidney Int*. 2003; 63:1313–20. [PubMed: 12631348]
18. Grover PK, Thurgood LA, Ryall RL. The effect of urine fractionation on the attachment of calcium oxalate crystals to renal epithelial cells: Implications for studying renal calculogenesis. *Am J Physiol Renal Physiol*. 2007; 292:F1396–F403. [PubMed: 17267387]
19. Kumar V, Yu S, Farrell G, Toback FG, Lieske JC. Renal epithelial cells constitutively produce a protein that blocks adhesion of crystals to their surface. *Am J Physiol Renal Physiol*. 2004; 287:F373–F83. [PubMed: 15100100]
20. Verkoelen CF, van der Boom BG, Romijn JC. Identification of hyaluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. *Kidney Int*. 2000; 58:1045–54. [PubMed: 10972669]

21. Yamaguchi S, Wiessner J, Hasegawa A, Hung L, Mandel G, Mandel S. Calcium oxalate monohydrate crystal binding substance produced from Madin-Darby canine kidney cells. *Int J Urol.* 2002; 9:501–8. [PubMed: 12410930]
22. Ryall RL, Fleming DE, Grover PK, Chauvet MC, Dean CJ, Marshall VR. The hole truth. intracrystalline proteins and calcium oxalate kidney stones. *Mol Urol.* 2000; 4:391–402. [PubMed: 11156707]
23. Ryall RL, Chauvet MC, Grover PK. Intracrystalline proteins and urolithiasis: a comparison of the protein content and ultrastructure of urinary calcium oxalate monohydrate and dehydrate crystals. *BJU Int.* 2005; 96:654–63. [PubMed: 16104927]
24. Lieske JC, Huang E, Toback GF. Regulation of renal epithelial cell affinity for calcium oxalate crystals. *Am J Physiol.* 2000; 278:F130–F7.
25. Chauvet MC, Ryall RL. Intracrystalline proteins and calcium oxalate crystal degradation in MDCK II cells. *J Struct Biol.* 2005; 151:12–7. [PubMed: 15941662]
26. Grover PK, Thurgood LA, Fleming DE, van Bronswijk W, Wang T, Ryall RL. Intracrystalline urinary proteins facilitate degradation and dissolution of calcium oxalate crystals in cultured renal cells. *Am J Physiol.* 2008; 294:F355–F61.
27. Lieske JC, Toback GF. Renal cell–urinary crystal interactions. *Curr Opin Nephrol Hy.* 2000; 9:349–55.
28. Hanein D, Sababay H, Addadi L, Geiger B. Selective interaction of cells with crystal surfaces. Implications for the mechanism of cell adhesion. *J Cell Sci.* 1993; 104:275–88. [PubMed: 8505361]
29. Hanein D, Geiger B, Addadi L. Differential adhesion of cells to enantiomorphous crystal surfaces. *Science.* 1994; 263:1413–6. [PubMed: 8128221]
30. Zimmerman E, Addadi L, Geiger B. Effects of surface-bound water and surface stereochemistry on cell adhesion to crystal surfaces. *J Struct Biol.* 1999; 125:25–38. [PubMed: 10196114]
31. Asselman M, Verhulst A, De Broe ME, Verkoelen CF. Calcium oxalate crystal adherence to hyaluronan-, osteopontin-, and CD44-expressing injured/regenerating tubular epithelial cells in rat kidneys. *J Am Soc Nephrol.* 2003; 14:3155–66. [PubMed: 14638914]
32. Verhulst A, Asselman M, Persy VP, et al. Crystal retention capacity of cells in human nephron: involvement of CD44 and its ligands hyaluronic acid and osteopontin in the transition of a crystal binding - into a nonadherent epithelium. *J Am Soc Nephrol.* 2003; 13:107–15. [PubMed: 12506143]
33. Kasemo B, Lausmaa J. Material–tissue interfaces. the role of surface properties and processes. *Environ Health Perspect.* 1994; 102(Suppl. 5):41–5. [PubMed: 7882954]
34. Riese RJ, Mandel NS, Wiessner JH, Mandel GS, Becker CG, Kleinman JG. Cell polarity and calcium oxalate crystal adherence to cultured collecting duct cells. *Am J Physiol.* 1992; 262:F177–F84. [PubMed: 1539682]
35. Wiessner JH, Hasegawa AT, Hung LY, Mandel GS, Mandel NS. Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells. *Kidney Int.* 2001; 59:637–44. [PubMed: 11168945]
36. Bigelow MW, Wiessner JH, Kleinman JG, Mandel NS. The dependence on membrane fluidity of calcium oxalate crystal attachment to IMCD membranes. *Calcif Tissue Int.* 1997; 60:375–9. [PubMed: 9075636]
37. Wiessner JH, Hasegawa AT, Hung LY, Mandel NS. Oxalate-induced exposure of phosphatidylserine on the surface of renal epithelial cells in culture. *J Am Soc Nephrol.* 1999; 10:S441–5.
38. Bigelow MW, Wiessner JH, Kleinman JG, Mandel NS. Calcium oxalate crystal attachment to cultured kidney epithelial cell lines. *J Urol.* 1998; 160:1528–32. [PubMed: 9751407]
39. Ghosh P, Katti DR, Katti KS. Mineral proximity influences mechanical responses of proteins in biological mineral – protein hybrid systems. *Biomacromolecules.* 2007; 8:851–6. [PubMed: 17315945]
40. Thurgood LA, Grover PK, Ryall RL. High calcium concentration and calcium oxalate crystals cause significant inaccuracies in the measurement of urinary osteopontin by enzyme linked immunosorbent assay. *Urol Res.* 2008; 36:103–10. [PubMed: 18478219]

41. Roy ME, Nishimoto SK. Matrix Gla protein binding to hydroxyapatite is dependent on the ionic environment: calcium enhances binding affinity but phosphate and magnesium decrease affinity. *Bone*. 2002; 31:296–302. [PubMed: 12151082]
42. Grover PK, Ryall RL. Effect of prothrombin and its activation fragments on calcium oxalate crystal growth and aggregation in undiluted human urine in vitro: relationship between protein structure and inhibitory activity. *Clin Sci*. 2002; 102:425–34. [PubMed: 11914105]
43. Goldberg HA, Warner KJ, Li MC, Hunter GK. Binding of bone sialoprotein, osteopontin and synthetic polypeptides to hydroxyapatite. *Connect Tissue Res*. 2001; 42:25–37. [PubMed: 11696986]
44. Langdon A, Wignall GR, Rogers K, et al. Kinetics of calcium oxalate crystal growth in the presence of osteopontin isoforms: an analysis by scanning confocal interference microscopy. *Calcif Tissue Int*. 2009; 84:240–8. [PubMed: 19189038]
45. Doyle IR, Ryall RL, Marshall VR. Inclusion of proteins into calcium oxalate crystals precipitated from human urine: a highly selective phenomenon. *Clin Chem*. 1991; 37:1589–94. [PubMed: 1893595]
46. Fleming DE, van Riessen A, Chauvet MC, et al. Intracrystalline proteins and urolithiasis: a synchrotron X-ray diffraction study of calcium oxalate monohydrate. *J Bone Min Res*. 2003; 18:1282–91.
47. Ryall RL, Grover PK, Thurgood LA, Chauvet MC, Fleming DE, van Bronswijk W. The importance of clean face: The effect of different washing procedures on the association of Tamm-Horsfall glycoprotein and other urinary proteins with calcium oxalate crystals. *Urol Res*. 2007; 35:1–14. [PubMed: 17277922]
48. Lieske JC, Farrell G, Deganello S. The effect of ions at the surface of calcium oxalate monohydrate crystals on cell–crystal interactions. *Urol Res*. 2004; 32:117–23. [PubMed: 14663633]
49. Rabinovich YI, Daosukho S, Byer KJ, El-Shall HE, Khan SR. Direct AFM measurements of adhesion forces between calcium oxalate monohydrate and kidney epithelial cells in the presence of Ca^{2+} and Mg^{2+} ions. *J Colloid Interface Sci*. 2008; 325:594–601. [PubMed: 18619606]
50. Doyle, IR.; Ryall, RL.; Marshall, VR. The effect of low speed centrifugation and Millipore filtration on the urinary protein content. In: Walker, VR.; Sutton, RAL.; Cameron, EC.; Pak, CYC.; Robertson, WG., editors. *Urolithiasis*. Plenum Press; New York: 1989. p. 593-5.
51. Maslamani S, Glenton PA, Khan SR. Changes in urine macromolecular composition during processing. *J Urol*. 2000; 164:230–6. [PubMed: 10840465]
52. Stapleton AMF, Ryall RL. Crystal matrix protein – Getting blood out of a stone. *Miner Electrolyte Metab*. 1994; 20:399–409. [PubMed: 7783703]
53. Stapleton AMF, Ryall RL. Blood coagulation proteins and urolithiasis are linked: Crystal matrix protein is the F1 activation peptide of human prothrombin. *Br J Urol*. 1995; 75:712–9. [PubMed: 7613825]
54. Fleming, DE. PhD Thesis. Curtin University of Technology; 2004. *Urolithiasis: Occurrence and Function of Intracrystalline Proteins in Calcium Oxalate Monohydrate Crystals*. Available at <http://www.adt.curtin.edu.au/theses/available/adtWCU20050124.09385/>
55. Aizenberg J, Hanson J, Ilan M, et al. Morphogenesis of calcitic sponge spicules: a role for specialized proteins interacting with growing crystals. *FASEB J*. 1995; 9:262–8. [PubMed: 7781928]
56. Cook AF, Grover PK, Ryall RL. Face-specific binding of prothrombin fragment 1 and human serum albumin to inorganic and urinary calcium oxalate monohydrate crystals. *BJU Int*. 2009; 103:826–35. [PubMed: 19021614]
57. Aizenberg J, Hanson J, Koetzle TF, Weiner S, Addadi L. Control of macromolecule distribution within synthetic and biogenic single crystals. *J Am Chem Soc*. 1997; 119:881–6.
58. Doyle IR, Marshall VR, Dawson CJ, Ryall RL. Calcium oxalate crystal matrix extract. The most potent macromolecular inhibitor of crystal growth and aggregation yet tested in undiluted urine *in vitro*. *Urol Res*. 1995; 23:53–62. [PubMed: 7618236]
59. Ryall RL. Urinary inhibitors of calcium oxalate crystallization and their potential role in stone formation. *World J Urol*. 1997; 15:155–64. [PubMed: 9228722]

60. Ryall RL. Macromolecules and urolithiasis: parallels and paradoxes. *Nephron Physiol.* 2004; 98:37–42.

Fig. 1.

The percentage attachment of the same sCOM (square), cfCOM (diamond), ufCOM (triangle) and iCOM (circle) crystals to MDCK-II cells, using six different UF urine samples as the binding medium.

Fig. 2. The median percentage attachment of the sCOM (square), cfCOM (diamond), ufCOM (triangle) and iCOM (circle) crystals, plotted as a function of the mean protein concentrations in a pure inorganic solution (protein concentration = 0 mg/L), and in 10 individual urine samples after being sieved, CF, or UF.

Fig. 3.

(a) Western blot, stained for PTF1, of the matrix extracts of CaOx crystals containing intracrystalline CME. Lane 1, molecular weight markers; lane 2, crystals grown in UF urine; lane 3, 0.05 mg/L CME; lane 4, 0.1 mg/L CME; lane 5, 0.5 mg/L CME; lane 6, 5.0 mg/L CME. (b) Western blot, stained for PTF1, of the matrix extracts of CaOx crystals containing intracrystalline + surface-bound CME. Lane 1, molecular weight markers; lane 2, crystals grown in UF urine; lane 3, 0.05 mg/L CME; lane 4, 0.1 mg/L CME; lane 5, 0.5 mg/L CME; lane 6, 5.0 mg/L CME.

Fig. 4. Effect of intracrystalline (circle) and intracrystalline + surface-bound (triangle) proteins on the attachment of COM crystals precipitated from undiluted, ultrafiltered urine containing CME at final protein concentrations of 0, 0.05, 0.10, 0.5 and 5 mg/L.

Fig. 5.

Diagrammatic representation of crystals associated with intracrystalline (top row) or surface-bound (bottom row) proteins, in approximate proportion to the concentrations of CME used to generate the crystals for the CME dose–response experiment shown in Fig. 4. At a CME concentration of 0 mg/L, no proteins are present either inside or on the surfaces of the crystals. As the ambient concentration of protein increases, the amount of protein included within the mineral also increases. However, because intracrystalline protein is concentrated at the crystal centre [22,56], at concentrations from 0.05 to 0.5 mg/L it is completely covered with COM mineral and has little or no effect on the surface properties of the crystal. When the protein concentration increases to 5.0 mg/L the outermost layer of protein molecules is partially embedded in the mineral phase, but also protrudes beyond the limit of mineral ions and hence, can influence binding of the crystal to the cell surface. In contrast, proteins attached to the surface of the crystal are always exposed and are more likely to affect attachment of the crystal to the cell surface, even at relatively low concentrations, as shown quantitatively in Fig. 4.