Effect of urine fractionation on attachment of calcium oxalate crystals to renal epithelial cells: implications for studying renal calculogenesis

Phulwinder K. Grover, Lauren A. Thurgood, and Rosemary L. Ryall

Urology Unit, Department of Surgery, Flinders Medical Centre, and Flinders University, South Australia, Australia

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Grover PK, Thurgood LA, Ryall RL. Effect of urine fractionation on attachment of calcium oxalate crystals to renal epithelial cells: implications for studying renal calculogenesis. Am J Physiol Renal Physiol 292: F1396–F1403, 2007. First published January 30, 2007; doi:10.1152/ajprenal.00456.2006.—Our aim was to determine whether fractionation of human urine affects the attachment of calcium oxalate monohydrate (COM) crystals to renal cells. Urine collected from six healthy subjects was fractionated into sieved (S), centrifuged (C), centrifuged and filtered (CF), or ultrafiltered (UF). Attachment of $[^{14}C]$COM crystals to Madin-Darby canine kidney (MDCK) cells was studied after precoating the crystals or the cells with the urine fractions and by using the same fractions as the binding medium. Protein content of the fractions and precoated crystals was analyzed with SDS-PAGE and Western blotting. All urine fractions inhibited crystal attachment. When fractions from the six urine samples were used to precoat the cells, the median inhibitions of crystal adhesion (~40%) were not significantly different. Median inhibition after preincubation of crystals was the same for the S, C, and CF fractions (~40%) but significantly greater than for the UF fraction (~28%). When fractions were used as the binding medium, median inhibitions decreased from 64% in the S fraction to 47 (C), 42 (CF), and to 29% (UF). SDS-PAGE analysis showed that centrifugation and filtration reduced the amount of Tamm-Horsfall glycoprotein (THG), which was confirmed by Western blotting. Human serum albumin, urinary prothrombin fragment 1, and osteopontin, but not THG, were present in demineralized extracts of the precoated crystals. Fractionation of human urine affects the attachment of COM crystals to MDCK cells. Hence future studies investigating regulation of crystal-cell interactions should be carried out in untreated urine as the binding medium.

Madin-Darby canine kidney cells; renal calculi

The discovery in the 1990s that crystals of calcium oxalate (CaOx), the predominant mineral phase of urinary calculi, irreversibly adhere to and are internalized by cultured renal epithelial cells (26, 30) paved a new path in urolithiasis research. Although it has always been acknowledged that calculogenesis requires the retention of nascent CaOx crystals within the kidney, the process by which this occurs has remained unclear. Advocates of a “free-particle” mechanism of stone formation propose that retention results from a combination of deposition of solute and aggregation of individual particles, a hypothesis that has been shown to be theoretically possible (16). Nonetheless, current opinion favors a “fixed-particle” mechanism, a term that originates from a theoretical treatise published in the late 1970s. Using known renal tubular dimensions, urine flow rates, and measured levels of urinary supersaturation, Finlayson and Reid (11) concluded that fixation or nucleation of crystalline particles on the luminal wall is necessary for the initiation of renal stone disease.

The fixed-particle concept proved to be the impetus for a long series of papers studying the binding of CaOx crystals to cultured renal cells, although, curiously, direct attempts to observe and quantify the phenomenon did not occur until 14 years later, when Lieske and colleagues (30) tested the binding of CaOx crystals to nontransformed monkey renal epithelial (BSC-1) cells. That paper was followed by intense research that continues today and that has generated a plethora of reports investigating factors regulating interactions between crystals and cultured renal cells (reviewed in Refs. 13, 21, 29). Those reports revealed that binding of CaOx monohydrate (COM) crystals, the more injurious (46) and more prevalent form of the mineral in human kidney stones (31), to renal epithelial cells is rapid, concentration dependent, and significant greater than that of other calcium crystals (24, 27, 28). Furthermore, crystals bind to anionic sites on the cell surface in a dynamic process that can be altered by manipulating the surface properties of the cells (22, 29). Adhesion is followed by internalization (25, 27, 28), which is a nephron segment-specific process (36). Although those observations prompted the conviction that crystal attachment and internalization encourage the development of stone disease, paradoxically, the same processes may also serve as a routine form of defense.

After internalization, crystals dissolve over a period of 5–7 wk in lysosomal inclusion bodies (21), which has generated the hypothesis that crystal attachment to the renal epithelial lining, together with the ensuing cellular responses, is a routine process that protects against calculogenesis (20, 21, 23, 25, 39, 42). However, the pathophysiological relevance of most published studies of crystal-cell interactions suffers to some degree by the fact that crystal binding was carried out using aqueous inorganic buffers, instead of urine, the real medium in which stones form. Since urine contains a spectrum of low- and high-molecular-weight constituents, the ratios of which vary from sample to sample even when collected from the same subject, it can be reasonably argued that no synthetic solution can possibly mimic the likely effects in urine. In only a handful of previous studies urine samples were used as a proxy for tubular fluid (10, 17, 18), but the samples were pretreated in different ways to remove cellular debris or concentrate the macromolecules. In one case (10), the urine was diluted to final concentrations of only 1 or 10%. It is therefore difficult to assess the effects of urinary macromolecules on the attachment of crystals to cells, especially when it has been well docu-
mented that prior treatment of urine by centrifugation and filtration significantly alters the profile of its macromolecules (6, 32). Therefore, the aim of this investigation was to determine whether fractionation of human urine affects the attachment of COM crystals to cultured renal cells.

MATERIALS AND METHODS

Urine collection and Fractionation

Twenty-four-hour urine samples were collected without preservative from six healthy laboratory colleagues who had no previous history of urinary stone disease. This study was reviewed and approved by the Committee on Clinical Investigation (Ethics Committee) of the Flinders Medical Centre.

The samples were refrigerated during the collection period and during storage before use. After confirming the absence of blood by dip stick analysis, the pH of the samples was adjusted to 6.11 and the samples were divided into four aliquots. The first portion was strained through a 70-μm sieve (S). The second was centrifuged (C) at 9,000 × g for 20 min at 20°C. The third was centrifuged as above and then filtered (CF) through a 0.22-μm Millipore filter. The fourth was centrifuged and filtered as above and then ultrafiltered (UF) through a regenerated cellulose cartridge (catalog no. CDUF001LC, Millipore, Bedford, MA) with a nominal relative molecular mass threshold of 10 kDa.

Generation of Radiolabeled Inorganic Crystals

Radioactive inorganic COM crystals were generated as described previously (4). They were sterilized by ethylene oxide, and immediately before use they were suspended in the various urine samples and sonicated for 10 min to disrupt any aggregates. Nonradioactive COM crystals were generated in an identical manner, but without sonication for 10 min to disrupt any aggregates. They were sterilized by ethylene oxide, and immediately before use they were suspended in the various urine samples and sonicated for 10 min to disrupt any aggregates. All experiments included a control comprising PBS saturated with CaOx (pH 6.11) to allow direct comparison of interexperimenral data. Inhibition of crystal attachment was calculated from the measured level of radioactivity in the cell supernatant after dissolution, relative to that contained in the original crystal suspension.

Effect on crystal attachment of precoating cells with S, C, CF, and UF urine fractions. Four milligrams of 14C-labeled COM crystals were placed in a Falcon tube containing 20 ml of S, C, CF, or UF urine and incubated in a rotary mixer for 1 h at room temperature. The crystals were pelleted by centrifugation at 2,000 × g for 5 min at 20°C and suspended in 25 ml of distilled water saturated with CaOx. The slurries were vigorously vortex-mixed and recentrifuged as above. This washing cycle was repeated nine times, after which the crystals were freeze-dried and resuspended in the UF fraction at a ratio of 400 μg crystals/2 ml urine. Binding of the precoated crystals to the monolayer was studied in the corresponding UF fraction, after the cells were washed three times with 2 ml of the same UF urine as outlined above.

Effect of urinary macromolecules on crystal-cell interactions.

Viability of Cells After Incubation in Urine

This was checked in preliminary studies using S, C, CF, and UF samples prepared from a single urine specimen. The culture medium was aspirated, and cells were rinsed three times with 2 ml of UF urine. Only UF urine was used for washing, since this enabled the cells to be conditioned to urinary conditions without coating them with any urinary macromolecules. Aliquots (2 ml) of the same UF urine and the corresponding S, C, and CF samples were then added to separate culture dishes before incubation at 37°C for varying periods of time up to 80 min. The cells were removed with a cell scraper, and their viability was checked using trypan blue exclusion and light microscopy and a hemocytometer. Approximately 97–98% cells were alive after 80 min, regardless of the urine fraction in which they had been incubated.

Kinetics of Crystal Attachment

To determine the time required to achieve maximum binding, a preliminary experiment was performed with one pooled urine specimen processed to give S, C, CF, or UF samples. The cells were rinsed as described above and then supplemented with 2 ml of S, C, CF, or UF urine in which had been suspended 400 μg of 14C-labeled COM crystals. The plates were gently agitated to distribute the crystals uniformly. After incubation of the dishes for periods of time up to 80 min, the urine was aspirated and the cells were washed three times with the same UF urine as described above. The attached crystals were dissolved by adding an additional 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 Ready Safe scintillation fluid (Beckman) in a liquid scintillation counter (Beckman LS 3801 Liquid Scintillation System).

Since preliminary studies had shown that the extent of crystal attachment in urine differed significantly from that in PBS at the same pH value of 6.11 (data not included), all experiments included a control comprising PBS saturated with CaOx (pH 6.11) to allow direct comparison of interexperimenral data. Inhibition of crystal attachment was calculated from the measured level of radioactivity in the cell supernatant after dissolution, relative to that contained in the original crystal suspension. Values were then expressed as a percentage of the values observed in the control incubation containing PBS, which was taken to be 0% inhibition.

Crystal Attachment Experiments

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before. They were incubated with 2 ml of S, C, CF, or UF urine for 30 min, followed by being washed three times with 2 ml of UF urine to remove any unattached urinary components. Two milliliters of the UF fraction containing 400 μg of [14C]-labeled crystals were then added to each monolayer, and binding was measured as described above.

Effect on crystal attachment of using different urine fractions as the binding medium. The culture medium was aspirated, and the cells were rinsed three times with 2 ml of the UF fraction of the urine sample. To each culture dish were added 2 ml of the S, C, CF, or UF urine fraction, in which was suspended (see above) 400 μg of [14C]-COM crystals, and the dish was gently agitated and incubated at 37°C for 15 min, since preliminary kinetic experiments had shown that maximum binding had occurred by that time. The urine was then aspirated, the cells were washed three times with UF urine, and the attached radioactivity was determined as detailed above.

Isolation of Proteins Bound to Surface of COM Crystals

Precoating and washing of unlabeled COM crystals were performed as described above. They were then demineralized using 0.25 M EDTA (pH 8.0) as described previously (7). The resulting extracts were desalted by extensive dialysis against distilled water at 4°C, lyophilized, and stored at −20°C for later analysis. Aliquots (25 ml) of the S, C, CF, and UF fractions of the same urine sample were included for comparison of protein content. These were extensively dialyzed against distilled water at 4°C and lyophilized.

SDS-PAGE Analysis and Western Blotting

Lyophilized samples were mixed with reducing sample buffer, electrophoresed, and the gel was stained with silver as described previously (12). For Western blotting, after electrophoresis proteins were electrophoretically transferred to nitrocellulose membrane and immunoblotted as described previously (33). The antibodies used were as in our earlier study (33) except that for Tamm-Horsfall glycoprotein (THG), 1:500 dilution of rabbit anti-human THG (kindly donated by Professor John Hoyer) and 1:2,000 goat anti-rabbit IgG HRP conjugate (no. 1706515, Bio-Rad); and for human serum albumin (HSA), 1:2,000 dilution of rabbit anti-human albumin. (no. 126584, EMD Biosciences, San Diego, CA) were used.

Statistical Analysis

All experiments were performed in triplicate. Although data are plotted as means ± SE, the variances are smaller than the symbols used for the means and therefore do not appear in the figures. Nonetheless, statistical comparisons between data sets were performed using the Wilcoxon signed-rank sum test, and a level of P < 0.01 was considered statistically significant.
RESULTS

Figure 1 shows SDS-PAGE of proteins in the S, C, and CF fractions of one of the urine samples used. UF urine is not included because no proteins were detected. Although a wide spectrum of proteins is visible in all fractions, their profiles differ markedly only in the high-molecular-weight range. There is a prominent band running at the top of the lane from the S urine fraction, which is very faint in the C lane and barely visible in the CF lane. Western blotting confirmed that the prominent band is THG. In Fig. 2A, which is stained for THG, the S urine fraction shows a large diffuse band at approximately ~97 kDa, which extends into a smear running from ~100 to 300 kDa. However, the staining intensity of both the smear and the discrete band is very much reduced in the C lane. No staining is visible in the lane corresponding to the CF fraction. Figure 2B shows the Western blots for the S, C, and CF urine fractions, stained for urinary prothrombin fragment 1 (UPTF1), which is present in all lanes migrating at ~31 kDa. The corresponding blots for HSA are presented in Fig. 2C, which shows as a single wide band running at ~60 kDa. Osteopontin (OPN) migrated as a smear running from ~48 to 70 kDa in all the samples (Fig. 2D) but extending to higher molecular masses in the S fraction.

Figure 3 shows that crystal attachment in the presence of the different urine fractions followed saturation kinetics. Irrespective of the urine fraction used as the binding medium, attachment reached a plateau by 10 min. Thus in all subsequent binding studies crystals were left in contact with the cells for 15 min before removal of unattached crystals and determination of bound radioactivity.

Figure 4 shows the effect of precoating crystals with the different urine fractions. It reveals that the median inhibitions resulting from incubation of crystals in the S (40%), C (39%), and CF (42%) fractions, while significantly higher than the PBS control value (0%), do not differ significantly from each other. However, ultrafiltration decreased binding inhibition to 28.3%. The Western blot analysis of demineralized extracts of the crystals preincubated in the S, C, and CF fractions showed it to contain UPTF1 (Fig. 5A), HSA (Fig. 5B), and OPN (Fig. 5C), but not THG.

Figure 6 shows inhibition of crystal-cell attachment resulting from pretreatment of the cells with the S, C, CF and UF urine fractions. Inhibition caused by S urine was 39%, which did not differ significantly from the values obtained when the cells were pretreated with C (37.5%), CF (37%), and UF urine (36.7%), respectively.

Figure 7 shows the inhibition of attachment of untreated COM crystals using the different urine fractions as the binding medium. The median value fell significantly (P < 0.01) from 64% in the S urine to 47% in the C urine. Inhibition in the CF urine was only slightly (42%), but nonetheless significantly (P < 0.01) lower than that in the C urine, while the lowest value of 29% (P < 0.01 compared with CF) was obtained in UF urine.

Figure 8 shows a plot of the median values (% inhibition) of crystal binding in S, C, CF, and UF fractions used in the present study vs. their % total protein concentrations. Centrifugation of sieved urine reduces the total protein concentration to 68%; filtration causes a further reduction to 52% (6), while ultrafiltration obviously decreases the concentration of proteins >10 kDa to zero.

DISCUSSION

Studies investigating factors that regulate interactions of crystals with cultured renal cells are of utmost importance in urolithiasis research. However, most of these studies have been performed in aqueous inorganic buffers and not in urine, the real medium in which stones form. In addition, of the studies that used urine as the proxy for tubular fluid (10, 17–18), they too pretreated urine in different ways to remove cellular debris or concentrate the macromolecules. This is very perplexing because prior treatment of urine has been documented to significantly alter its macromolecular content in general and THG, HSA, and inter-α-inhibitor in particular (6, 32). This, in turn, can potentially change the milieu and perhaps attachment of crystals to cells. Therefore, the present investigation was undertaken to determine whether fractionation of human urine affects the attachment of COM crystals to cultured renal cells. This was achieved by precoating either the crystals or the cells with different urine fractions and by using the same fractions as the medium in which the binding experiments were carried out. Cell attachment studies were combined with qualitative anal-
yses of several proteins implicated in stone formation, namely, THG, HSA, UPTF1, and OPN.

Initial studies investigating protein profiles of S, C, and CF fractions showed them as differing markedly only in the high-molecular-weight range. Western blotting suggested that their amounts of HSA, UPTF1, and OPN are largely unaffected by fractionation since their band intensities were approximately the same in all lanes. However, it did affect THG, which was significantly reduced in the C and absent from the CF fraction. No attempt was made in the present study to quantify the effect of processing of urine on individual urinary proteins.

Fig. 5. Western blots of proteins present in the demineralized extracts of COM crystals preincubated in the S, C, and CF fractions of one of the urine samples shown in Fig. 4 stained for UPTF1 (A), HSA (B), and OPN (C).

Fig. 6. Effect of preincubation of cells with S (○), C (■), CF (▲), and UF (●) fractions of the same urine samples the data of which are presented in Fig. 4.

Fig. 7. Effect of using as binding medium the S (○), C (■), CF (▲), and UF (●) fractions of the same urine samples the data of which are presented in Figs. 4 and 6.
proteins, first, because we (6) and others (32) have previously measured changes in HSA and THG, and Maslamani et al. (32) have shown changes in a number of other proteins; and second, because our primary aim was to observe the effects of urine fractionation on crystal-cell attachment. Collectively, therefore, the results presented here and elsewhere demonstrate that fractionation of urine causes a reduction in both the amount and type of proteins, decreasing in the order S > C > CF > UF.

Kinetics of attachment of COM crystals to cells in the presence of the different urine fractions showed, as have been reported previously with BSC-1 (24, 27–28) and MDCK (16, 27–28) cells, that crystal adhesion was very rapid, with significant attachment occurring within 1 min and reaching a plateau by 10 min. Binding times in this study differed from those in previous reports, some of which also differed from each other (3, 8, 23–25, 41, 48), probably as a consequence of the use of different cell types, culture supports, and incubation media.

Attachment of precoated crystals showed that the median inhibitions resulting from incubation of crystals in the S, C, and CF fractions did not differ significantly from each other. However, ultrafiltration significantly decreased binding inhibition. All crystals had been washed copiously with distilled water, which removes any proteins loosely associated with the crystal surfaces (34). Thus it may be assumed that dissolved proteins bound tightly to the crystal surfaces and that are not present in the UF fractions are responsible for the difference in inhibition caused by the S, C, and CF fractions and that of the corresponding UF fraction, namely, ~12% (~40–28%). This was supported by Western blotting. The demineralized extracts of the crystals preincubated in the S, C, and CF fractions showed the presence of UPTF1, HSA, and OPN, but not THG. We have previously demonstrated that both OPN and UPTF1 bind to COM crystals generated from ultrafiltered urine and then incubated in filtered urine (33): UPTF1 binds more avidly at a lower (2 mM) calcium concentration, while OPN binds only at a higher (7 mM) concentration, which is consistent with the detection of OPN in the current investigation in which the calcium concentration was 5.5 mM. Thus pretreatment of COM crystals with urinary macromolecules inhibits their binding to cultured renal cells. This corroborates the results of other studies reporting that coating of the crystal surface with OPN (24), a highly acidic region of nucleolin-related protein (37), unidentified 200 (47)- and 39-kDa (19) proteins, bikunin (9), and fibronectin (38) inhibits COM crystal adhesion to cultured renal cells.

The question, however, remains as to whether macromolecules can inhibit crystal adhesion by binding to the cell surface. To explore this, crystal attachment resulting from pretreatment of the cells with the S, C, CF, and UF fractions was examined. Inhibition caused by S urine did not differ significantly from the values obtained when the cells were pretreated with C, CF, and UF urine, respectively. This suggests that macromolecules in urine, particularly THG, do not inhibit attachment by binding irreversibly to the cells. Furthermore, inhibition occurred in every instance, which demonstrates that the attachment of crystals to cells is also inhibited by urinary components other than macromolecules, since attachment was inhibited even in UF urine. Components of UF urine that could inhibit crystal attachment might include fragments of proteins and other calcium-binding macromolecules of <10 kDa, for instance, osteocalcin (15). In addition, urine contains calcium-binding amino acids, including aspartic and glutamic acids, as well as other low-molecular-weight species that have been shown to reduce the adhesion of COM crystals to renal cells, including prostaglandins (22) and citrate (24), which is present in urine at considerably higher molar concentrations than any single macromolecule.

Attachment of untreated COM crystals using the different fractions as the binding medium showed that the inhibition of crystal binding decreased significantly and consistently in the order S > C > CF > UF. This trend cannot be attributed to different extents of cellular damage caused by each fraction since, as mentioned above, cell viability was the same in every case. Equally, the progression does not result from differences in acute ionic effects of urine on cells, such as osmotic stress, which has been previously suggested to influence crystal binding (41), because the osmolality (which is determined principally by the total concentration of dissolved salts) was the same in all fractions.

Furthermore, the results showed that the largest changes in inhibition of crystal binding observed corresponded with fractionation steps that produce the greatest differences in protein concentration. This suggests that, of the macromolecules in urine, proteins are the most critical determinants of crystal binding, accounting for approximately half of the total observed inhibition in the S fraction. However, it is also apparent, as was noted above, that low-molecular-weight components of urine account for the remaining inhibition (~30%) occurring in the UF sample. Nonetheless, THG must account for a high proportion of the macromolecular-inhibitory activity, particularly that of the S fraction. The urinary concentration of THG in sieved urine is drastically reduced by centrifugation and filtration (6, 32). The absence of THG from the CF fraction demonstrates that most of the protein in the sieved fraction is particulate. This is consistent with the observation that centrifugation of sieved urine causes a large reduction (91.2%) in the number of particles, while filtration removes the remaining 8.8% (6), and confirms early observations that THG in normal urine exists as very large polymeric structures that are easily removed by centrifugation and filtration (5, 43–44), as well as more recent reports that levels of THG and other proteins are...
reduced by these procedures (6, 32). Thus the high inhibitory effect of the S urine fractions is almost certainly ascribable to the fact that S urine contains the highest concentration of polymerized particulate THG. This likelihood is considerably strengthened by the observation that crystals bound with equal affinity to cells that were pretreated with S, C, CF, or UF urine and subsequently washed with UF urine, which showed unambiguously that THG does not bind tightly to the cell surface. Furthermore, even if THG binds weakly, the fact that it was so easily removed by washing indicates that it is unlikely to remain bound in vivo. Renal epithelial cells are continuously bathed in fresh urine and are not arrayed on a planar surface, which would have maximized the possibility of gravitational settling and binding in the present investigation. Thus we conclude that the inhibitory effect of the S fraction results from its high content of suspended gelatinous THG (6), in which the crystals become trapped, or which deposits passively onto the monolayer, either or both of which could physically impede the attachment of crystals to the cell surfaces. In contrast, given their lack of effect when applied to the cells, inhibition caused by the C and CF fractions must result from the direct binding of dissolved macromolecules to the crystal surfaces.

THG has been reported to inhibit the internalization of COM crystals by MDCK and BSC-1 cells by interacting with the cells, rather than with the crystals (28). It has also been shown to have no effect on the attachment of COM crystals to cells (24), although later investigation by the same group (17, 18) and others (14) reported quite the opposite. Those studies (14, 24, 28) used aqueous solutions of human THG purified from urine, which were either added to the cell-binding medium or applied directly to the crystals. Similar studies have used COM crystals that had been incubated in a mixture of urinary macromolecules. One of these failed to show any effect (35), possibly because of the use of a large mass-to-volume ratio of crystals to urine may have caused insufficient surface coverage to achieve inhibition of attachment (17). Alternatively, the lack of effect may have resulted from mechanical injury inflicted to induce crystal adhesion (39). Attachment increases in response to scrape injury, as does the synthesis of hyaluronan (1), which mediates the attachment of crystals to injured cells (2, 40) and may therefore have overwhelmed any inhibition that urinary macromolecules may otherwise have caused. Other studies have reported that incubation of crystals in urinary macromolecules causes a reduction in crystal binding (17–18, 46). Because THG was the major protein associated with the crystals in those studies, the authors concluded that, by coating the crystal surface, THG plays a key role in preventing crystal attachment, either as an inhibitor (17–18, 45) or as a platform for the adsorption of other inhibitory macromolecules (45). However, we have recently shown, using a combination of SDS-PAGE, Western blotting, and synchrotron X-ray diffraction, that THG does not adsorb to the surface of either COM or COD (34) and that its detection and inhibitory effect on crystal binding reported in previous studies (17, 18, 45) were almost certainly caused by insufficient washing to ensure its complete removal from the crystals.

Taken together, the results presented here confirm that different fractions of urine contain different amounts and types of macromolecules and show that they inhibit COM attachment to cultured renal cells to different extents. Therefore, future studies investigating factors that regulate interactions of crystals with cultured renal cells should be carried out using untreated urine as the binding medium. Furthermore, PBS should be routinely included in crystal-binding experiments to account for intra- and interexperimental variation and to enable direct comparison of data from different sources.

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GRANTS

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