Modulation of Proliferating Renal Epithelial Cell Affinity for Calcium Oxalate Monohydrate Crystals

GERARD FARELL,* ERICK HUANG,† SOO Y. KIM,† RÜDIGER HORSTKORTE,‡ and JOHN C. LIESKE*

*Division of Nephrology, Mayo Clinic College of Medicine, Rochester, Minnesota; †Department of Medicine, The University of Chicago, Chicago, Illinois; and ‡Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Berlin, Germany

Abstract. Adhesion of urinary crystals to distal tubular cells could be a critical event that triggers a cascade of responses ending in kidney stone formation. Monolayer cultures of distal nephron-derived MDCKI cells were used as a model to study crystal–cell interactions. COM crystal adhesion reached a peak 2 d after plating and progressively fell thereafter. The decline in crystal binding was accelerated by prostaglandin E2 (PGE2) supplementation and delayed by blockade of PG production. Crystals avidly adhered to cells that migrated in to repair a scrape wound made in the monolayer and after a transient hypoglycemic insult. Exposure of MDCKI cells to uric acid crystals and soluble uric acid was also associated with increased crystal adhesion. Treatment of physically or hypoglycemically injured cells with trypsin or neuraminidase reduced crystal binding to baseline levels, suggesting that increased exposure of cell surface glycoproteins mediated the effect, whereas PGE2 treatment blunted crystal binding to regenerating cells. Furthermore, when cells were grown in the presence of synthetic d-mannosamine analogues that can modify the conformation of cell surface sialoglycoconjugates, crystal binding to proliferating cells was decreased, whereas blockade of N-glycosylation with tunicamycin increased crystal adhesion to these cells. Therefore, COM crystal binding is enhanced to growing renal cells, synthesis of N-glycosylated cell surface proteins is essential to downregulate crystal binding to cells, and this response is modulated by physiologic signals such as PGE2. Sialic acid residues seem to mediate crystal adhesion to growing cells, either directly or via linkage to other crystal-binding molecules. Subtle renal injury and subsequent nephron repair could be a factor promoting crystal adhesion and favoring calculus formation.

Urine is usually supersaturated with calcium and oxalate ions that nucleate to form calcium oxalate crystals (1). Unless these small crystals grow large enough to occlude a tubule lumen, aggregate with other crystals to form a mass large enough to do so, or adhere to the tubular epithelium, they will be swept out of the nephron in the flowing fluid within a few minutes, and kidney stones will not form. Indeed, calculations based on the rate of fluid flow and time required for crystals to nucleate from ions in tubular fluid suggest that an individual crystal will pass into the urine before it grows large enough to occlude the lumen and be retained in the nephron (2,3).

Recent evidence suggests that in many calcium oxalate stone formers, the earliest changes may be depositions of calcium phosphate in the medullary interstitium, that then serve as a nidus for a calcium oxalate stone (4). The processes that mediate calcium phosphate deposition and its evolution into calcium oxalate stones remain to be determined. In more marked hyperoxaluric states (e.g., enteric or primary hyperoxaluria), direct adhesion of calcium oxalate crystals to renal epithelial cells may predominate (4). Therefore, we and others have hypothesized that attachment of newly formed crystals to the tubular cell surface (5–10) and the cellular responses that follow (11–13) could result in crystal retention and thereby set in motion a series of events that lead to pathologic renal calcification. Adhesion of calcium oxalate crystals to anionic, sialic acid–containing molecules on the surface of renal epithelial cells is crystal-face specific (14) and can be blocked by competing soluble anions in tubular fluid such as glycosaminoglycans, citrate, or glycoproteins (5,6). Exposure of cells to agents that raise intracellular cAMP, including prostaglandin E2, (PGE2), decreases cellular affinity for crystals, whereas blockade of PGE production enhances it (15).

It was reported recently that COM crystals avidly adhere to renal cells that are migrating in to repair a scrape wound made in a monolayer (16), and hyaluronic acid has been proposed to mediate this phenomenon (17). In addition, studies in stone-forming humans (18,19) and oxalate-loaded rats (20) have demonstrated that renal cellular injury occurs. In the current study, we defined the effect of diverse injuries to renal cells on crystal adhesion and how the PGE system might modulate this response.

Materials and Methods

Cell Culture

Renal epithelial cells of the MDCK line, type I, were a gift of Carl Verkoelen (Erasmus University, Rotterdam, The Netherlands) and
were grown in Dulbecco-Vug modified Eagle's medium that contained 25 mM glucose (DMEM) at 38°C in a CO2 incubator as described previously (13). For preparing high-density, quiescent cultures, 1 × 10^6 cells/35-mm plastic plate (9.62 cm²; Nunc, Naperville, IL) were plated in DMEM that contained 10% calf serum and 1.6 mM biotin. Two days later, when they were confluent, the medium was aspirated and replaced with fresh medium that contained 5% calf serum and 1.6 mM biotin. The monolayer was used for study the next day. For time points beyond 3 d, medium was replaced daily with fresh medium that contained 5% calf serum and 1.6 mM biotin.

For evaluating their effect on adhesion of crystals to cells, PGE₂ (1.0 μM), the nonsteroidal anti-inflammatory agent flurbiprofen (10 μM), or the N-glycosylation inhibitor tunicamycin (50 ng/ml) was added directly to the culture medium and replenished each time the medium was changed. An equal volume of the vehicle (ethanol) was added to control cultures.

**Scrape Wounding of Monolayers**

For creating scrape wounds in an MDCKI monolayer, 5 d after plating, a 200-μl plastic pipette was used to remove carefully a line of cells ~2 to 3 mm wide across the entire width of the culture dish. Four wounds were made in each culture dish as two sets of parallel wounds were made on each coverslip. Two perpendicular wounds were made in each culture dish as two sets of parallel wounds were made on each coverslip.

For quantifying the rate of wound healing, the margins of a wound were marked carefully in four places on the outside bottom of the plastic dish using a razor blade. On each plate, one region was marked on each of the four wounds, creating a square with dimensions equal to the wound width. At baseline and daily after wounding, the wound width was measured in the marked region using an eyepiece reticle, and the number of cells that had migrated into the square was counted.

For experiments to evaluate the effect of coating with annexin V (PharMingen, San Diego, CA), cells were grown on glass coverslips (Fisher brand coverslips for growth; Fisher Scientific, Pittsburgh, PA). For creating scrape wounds on the coverslips, 5 d after plating, a dissecting needle was used to remove carefully a line of cells ~2 to 3 mm wide across the entire width of the coverslip. Two perpendicular wounds were made on each coverslip.

**Hypoglycemic Challenge of Monolayers**

For modeling a transient hypoglycemic insult, as might occur during a period of decreased renal perfusion, confluent MDCKI monolayers were preincubated for 3 h in glucose-free DMEM that contained 10% calf serum (21). After that, the medium was replaced with glucose-free DMEM that contained 10 μM antimycin A, 10 mM 2-deoxyglucose, and 10% calf serum for 1 h. Cells were then returned to DMEM that contained 10% calf serum, and thereafter the medium was replaced daily with fresh medium that contained 10% calf serum.

**Uric Acid and Uric Acid Crystal Treatment of Monolayers**

For exposing cells to uric acid (UA) crystals, the medium of confluent MDCKI monolayers was replaced with a low-sodium, low-pH buffer (141 mM choline chloride, 1.27 mM CaCl₂, 5.36 mM KCl, 0.44 mM KH₂PO₄, 5 mM glucose, 0.81 mM MgSO₄, 0.34 mM Na₂HPO₄, and 10 mM HEPES [pH 5]) that contained minimal essential vitamins, amino acids, and 5% calf serum (22), to which 800 μg/ml crystals was added. Two hours later, the buffer was aspirated and replaced with DMEM that contained 5% calf serum; thereafter, the medium was replaced daily with fresh medium that contained 5% calf serum. For exposing cells to soluble UA, the medium of confluent MDCKI monolayers was replaced with PBS (10 mM Na₂PO₄, 155 mM NaCl, 5.4 mM KCl [pH 7.4]) that contained physiologic concentrations of glucose (25 mM) and specified concentrations of UA (0.01 to 0.5 mg/ml). One hour later, the buffer was aspirated and replaced with DMEM that contained 5% calf serum.

**Enzymatic Treatment of Cells**

For assessing the contribution of cell surface molecules to adhesion of COM crystals, monolayer cultures of MDCKI cells were incubated with either neuraminidase (1 U/ml [pH 5], 1 h) or trypsin (50 μg/ml [pH 7.4], 15 min) in Hank's buffered salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose) at 38°C as described previously (5). Preliminary experiments defined these enzyme concentrations as having maximal effects on crystal binding without being cytotoxic. Hank's buffered salt solution that contained the enzyme of interest was then aspirated and replaced with 2 ml of PBS to which [¹⁴C]COM crystals were added as described below.

**Sialic Acid Precursor Analogues**

Synthetic α-mannosamine analogues with elongated aliphatic N-acyl groups can act as precursors for biosynthetically modified sialic acids (23). N-propenyl (ManNProp) and N-pentenyl α-mannosamine (ManNPent) were synthesized at Freie Universität Berlin (R.H.); N-acetyl α-mannosamine (ManNAc) was purchased from Sigma Chemical Co. (St. Louis, MO). Each sialic acid precursor (5 mM) was added to the culture medium at the time MDCKI cells were plated in DMEM that contained 10% calf serum (23). Two days after plating, the medium was replaced with fresh DMEM that contained 10% calf serum and the same precursor. Crystal binding was assessed 3 and 5 d after plating. In studies with scrape-wounded monolayers, sialic acid precursors were added only after wounding (day 5 after plating). Crystal adhesion was quantified 2 d later.

**Adhesion of Crystals to Cells**

To measure adhesion of crystals to cells, culture medium was aspirated and replaced with 5 ml of PBS at 38°C. [¹⁴C]COM crystals were added to the buffer to achieve a final concentration of 200 μg/ml (41.6 μg/cm² cells) from sterile slurry in distilled water that was constantly stirred at 1500 rpm to prevent aggregation. The culture dishes were agitated gently for 5 s to distribute uniformly the crystals that then settled to the surface of the cell monolayer under the force of gravity. After 2 min, buffer was aspirated and the cells were washed three times with PBS (5 ml). The cells were then scraped directly into a scintillation vial that contained 6 N HCl (0.5 ml) to which 4.5 ml of Ecoscint (National Diagnostics, E. Palmetto, FL) was added, and the amount of radioactivity was measured (7).

**Materials**

Crystals of COM were prepared from supersaturated solutions by Y. Nakagawa (University of Chicago) as reported previously (24). For preparing radioactive COM crystals, [¹⁴C]oxalic acid (30 to 60 mCi/ mmol; ICN Biomedicals, Irvine, CA) was added to a sodium oxalate solution, producing a specific activity of 10⁷ cpm/ml, and sufficient calcium chloride was then added to form a supersaturated solution. The COM crystals that precipitated had a range in specific activity from 300 to 450 cpm/μg. Therefore, baseline crystal adhesion values could vary as much as 50%. Crystal size and shape were assessed by light and scanning electron microscopy (11). COM crystals were cuboidal to spindle shaped and uniformly small at 1 to 2 μm in largest diameter. Crystals were sterilized by heating to 180°C overnight. X-ray crystallography performed by S. Deganello (University of Pal-
ermo, Italy) demonstrated that heating did not alter the structure of COM crystals.

UA crystals were prepared by Y. Nakagawa by dissolving 100 mg of UA in 250 ml of hot (60°C) distilled water to which ethanol (250 ml) was added, after which the solution was allowed to cool to room temperature with stirring overnight (25). The resulting crystal suspension was filtered, washed twice with ethanol, washed twice with acetone, and finally air-dried. The crystals that formed were planar and 2 to 10 μm in size, similar to crystals that can be seen in urine. Reagents were purchased from Sigma Chemical Company, unless otherwise indicated.

**Statistical Analyses**

Data were compared by t test; \( P < 0.05 \) was accepted as significant. Values presented are means ± SEM. When no measure of variance appears on a graph, it is because the variance is smaller than the symbol used for the mean. Points on graphs are each the mean of six or more values (\( n = 3 \) for each experiment performed at least twice).

**Results**

**Time Course of PGE\(_2\) Effect on COM Crystal Adhesion to MDCKI Cells**

After attaining confluence 2 d after plating, crystal binding to MDCKI cells fell progressively so that by day 7, crystal adhesion was only 2% of that on day 2 (Figure 1). When PGE\(_2\) (1.0 μM) was present in the medium from the time of plating, crystal adhesion was lower than control each day and reached minimal baseline levels by day 5, 1 d earlier than control cells not supplemented with PGE\(_2\) (Figure 1A). Conversely, when the nonsteroidal anti-inflammatory agent flurbiprofen (10 μM) was present in the medium each day after plating to prevent PG production, crystal adhesion was greater than control until day 6, when adhesion to control and PGE\(_2\)- and flurbiprofen-treated cells became equal. When tunicamycin (50 ng/ml) was added to cultures on days 0, 1, 2, or 3, the fall in binding on days 3, 4, and 5 was ablated, and in fact crystal binding increased on later days (Figure 1B). Therefore, it seems that synthesis of complex N-glycosylated components of the glycocalyx is crucial to block crystal binding at these later times. Once crystal binding had achieved low levels (day 4 or later), tunicamycin had a lesser effect. Addition of the O-glycosylation inhibitor benzyl-2-acetimido-2-deoxy-α-D-galactopyranoside (0.5 mM) between days 0 and 3 modestly increased crystal binding to cells on subsequent days in a manner similar to tunicamycin (data not shown), suggesting that O-glycosylation of carbohydrate chains may also contribute to the fall in crystal binding. *In vivo*, intrarenal PGE could play an especially important role to decrease adhesion of crystals to any proliferating cells that are present.

**Scrape Wounding of Monolayers**

For simulating a physical wound as might occur along the nephron *in vivo*, for example after detachment of dead or dying cells, scrape wounds were made in confluent MDCKI monolayers as described in the Materials and Methods section.

**Figure 1.** Effect of prostaglandin E (PGE) and N-glycosylation inhibition on COM crystal adhesion to canine kidney epithelial cells of the MDCKI line. Cultures were prepared as described in the Materials and Methods section. The medium was supplemented with PGE\(_2\), flurbiprofen, or vehicle on the day cells were plated (day 0) and at the time the medium was changed (days 1 through 6). The affinity of cells for crystals was assessed by replacing the medium with PBS and measuring the amount of exogenous [\(^{14}\)C]COM crystals in PBS that bound to cells during a 2-min period. Crystal adhesion to control cultures progressively fell and reached very low levels 7 d after plating (A). Adhesion of crystals to cells that were exposed to PGE\(_2\) (1.0 μM) was less than control each day after plating and reached low baseline levels by day 5. Adhesion of crystals to cells in which PG production was blocked with flurbiprofen (10 μM) was markedly enhanced, especially during the first 4 d after plating. When tunicamycin (50 ng/ml) was added to cultures on days 0, 1, 2, or 3, crystal binding did not fall on days 4 and 5 (B). However, when added on day 4, tunicamycin had little effect. *P < 0.001 (A) or *P < 0.05 (B) versus control.
Crystal adhesion was markedly enhanced the day after wounding and remained elevated for 4 more days (Figure 2A). Examination under light and polarizing microscopy confirmed that the increased crystal binding was to cells that migrated in to repair the wounds (Figure 3). When the medium was supplemented with PGE₂, crystal adhesion to the migrating cells was diminished so that binding to the entire monolayer was close to control levels (Figure 2B). Conversely, when PG production was blocked with flurbiprofen, crystal binding to the migrating cells was markedly enhanced. Examination under light and polarizing microscopy confirmed that the enhanced crystal binding after scrape-wounding was to cells that migrated in to repair the wounds, and not to the rest of the monolayer (Figure 3). Therefore, crystals adhere avidly to migrating, proliferating cells that are repairing a wound made in a confluent monolayer. Crystal affinity to these proliferating, migrating cells is decreased by N-glycosylation of cell-surface molecules and by the presence of PGE.

We next investigated mechanisms that might mediate adhesion of crystals to the migrating cells. Flurbiprofen enhanced the rate of wound closure by nearly a full day (Figure 4A), even though the cyclo-oxygenase inhibitor markedly increased crystal binding (Figure 2B). PGE₂ supplementation did not alter the rate of wound closure, even though it markedly diminished crystal binding (Figure 2B). Therefore, it does not seem that the presence or absence of PGE alters crystal adhesion to migrating cells by changing the rate of wound closure. Next we treated wounded monolayers with neuraminidase or trypsin as described in the Materials and Methods section.

Figure 2. Effect of wounding on COM crystal adhesion to MDCKI cells. Cultures were prepared as described in the Materials and Methods section. On day 5, when crystal adhesion had achieved low baseline levels (see Figure 1), four scrape wounds were made in the monolayers. For the next 4 d, crystal binding to cells that migrated in to repair the wounds was enhanced (A). Supplementation of the medium with PGE₂ (1.0 μM) diminished adhesion of crystals to the migrating cells, whereas blockade of PG production with flurbiprofen (10 μM) markedly enhanced it (B). *P < 0.001 versus control (A) or wounded (B).

Figure 3. COM crystal adhesion to migrating, proliferating cells. Cultures were prepared as described in the Materials and Methods section. On day 5, when crystal adhesion had achieved low baseline levels, scrape wounds were made in the monolayers, and the medium was supplemented with flurbiprofen (10 μM) to block PG production. Two days later, crystal adhesion was markedly enhanced to cells that migrated in to repair the wounds, whereas binding remained low to the remainder of the monolayer. A region of the denuded wound is shown (*), with an area of established monolayer at the top right (@). Crystals appear dark black (arrow). Magnification, ×200.
Treatment with both enzymes returned crystal binding to control levels (Figure 4B). Examination under light microscopy confirmed that enzymatic treatment under the conditions that we used did not cause detachment of the migrating cells.

Phosphatidyl serine (PS) has been implicated as a crystal binding receptor when exposed on the outer plasma membrane of cells under experimental conditions (26). However, precoating migrating cells with annexin V, which binds to PS, did not alter crystal binding (Figure 4B). In addition, fluorescence-tagged Annexin V (PharMingen) did not stain migrating cells. Therefore, PS does not seem to mediate adhesion of COM crystals to cells that repair scrape wounds in a monolayer.

We also defined the time required for recovery of crystal adhesion after migrating cells were treated with trypsin. Monolayers were wounded and treated with flurbiprofen to block endogenous PGE production and thereby enhance crystal adhesion to migrating cells. Two days after wounding, treatment with trypsin abolished the increase in crystal adhesion as expected (Figure 4C). Four hours after enzymatic treatment, crystal adhesion had increased by 170%, and by 24 h, binding was the same as to untreated, wounded cultures. Therefore, these molecules that bind crystals and are removed by trypsin from the surface of migrating cells are progressively replaced over 24 h in culture.

When tunicamycin was present before wounding, crystal adhesion to proliferating, migrating cells did not increase to the same extent (Figure 4D). However, when added the day of or the day after wounding, crystal binding was increased. *P < 0.001 versus control (A, B, and D) or trypsin-treated (C).

Figure 4. Characteristics of cell-surface crystal binding molecules on proliferating and migrating MDCKI cells. Cultures were prepared and wounded as described in Figure 2. Supplementation of the medium with flurbiprofen (10 μM) accelerated the rate of wound healing, whereas PGE₂ (100 μM) had no significant effect (A). Two days after wounding, treatment of monolayers with neuraminidase (NA) or trypsin returned crystal binding to control, baseline levels (whereas precoating cells with annexin V had no effect; B). Crystal adhesion to wounded, trypsin-treated cells increased within 4 h after enzyme exposure and returned to the same level as wounded cultures that had not been treated with the enzyme within 24 h (C). When tunicamycin was present 2 d before wounding, the increase in binding to proliferating, migrating cells was blunted (D). However, when added the day of or the day after wounding, crystal binding was increased. *P < 0.001 versus control (A, B, and D) or trypsin-treated (C).
subsequent days. Therefore, the effects of tunicamycin are complex, perhaps because the inhibitor decreases the expression not only of crystal binding molecules but also of other carbohydrates that can block crystal binding to cells (see also Figure 1B).

**Hypoglycemic Challenge of Monolayers**
As another model of an injury that might occur along the nephron in vivo, perhaps during periods of relative hypoperfusion, we exposed cells to a transient hypoglycemic insult. Cultures were prepared and exposed to a hypoglycemic insult as described in the Materials and Methods section. Crystal adhesion was enhanced 2.7-fold immediately after a 1-h hypoglycemic challenge and returned close to baseline by 24 h (Figure 5A). A second increase in crystal adhesion occurred 3 d after the hypoglycemic challenge, and binding returned to baseline after 2 additional days. Supplementation with flurbiprofen diminished crystal binding at all time points between 0 and 72 h (B). Blockade of PG production with flurbiprofen enhanced crystal binding at all time points between 0 and 72 h (B). Treatment of monolayers with NA or trypsin 4 or 72 h after the ischemic challenge returned crystal binding to control, baseline levels (C). *P < 0.001 versus control (A) or ischemic challenge (B).

![Figure 5](image-url)  
**Figure 5.** Effect of hypoglycemia on COM crystal adhesion to MDCKI cells. Cultures were prepared as described in the Materials and Methods section. Three days after plating, cells were exposed to glucose-free DMEM for 3 h, followed by glucose-free DMEM that contained 10 μM antimycin A and 10 mM 2-deoxyglucose for an additional hour. Cells were then returned to standard DMEM that contained 10% calf serum. Crystal adhesion was markedly enhanced 4 h after the ischemic challenge and again 3 d later (A). Supplementation of the medium with PGE₂ diminished crystal binding to ischemically challenged cells at 72 h but not at earlier time points (B). Blockade of PG production with flurbiprofen enhanced crystal binding at all time points between 0 and 72 h. Treatment of monolayers with NA or trypsin 4 or 72 h after the ischemic challenge returned crystal binding to control, baseline levels (C). *P < 0.001 versus control (A) or ischemic challenge (B).
profen after the hypoglycemic challenge further enhanced crystal adhesion, especially 48 to 72 h later (Figure 5B). Addition of PGE₂ did not alter crystal binding to the injured cells in the early time period but partially ameliorated the second peak of increased binding 72 h later (Figure 5B). Treatment with trypsin or neuraminidase 4 h or 3 d after the hypoglycemic challenge abolished the increased binding (Figure 5C). Therefore, MDCKI cell crystal binding increases both immediately after a hypoglycemic challenge and several days later. In the intact kidney, even a transient period of hypoxia could predispose cells to enhanced crystal binding for a prolonged period.

**Exposure of Cells to UA Crystals and Soluble UA**

We recently characterized the capacity of renal cells to bind UA crystals (27). Given the association of hyperuricosuria and calcium oxalate stones (28) and the dramatic cellular response to UA crystals described by Emmerson and colleagues (22,29), we wondered what effect UA crystals might have on COM crystal binding to renal cells. The medium of confluent MDCKI monolayers was replaced with low-sodium, low-pH buffer to which 800 μg/ml crystals was added. Exposure of cells to the low-pH buffer did not alter crystal binding, but COM crystal adhesion increased by 32% 2 to 6 h after exposure to UA crystals, remained elevated 24 h later, and returned to baseline the following day (Figure 6A). Therefore, transient exposure of renal cells to UA crystals increases the likelihood of subsequent COM crystal adhesion. Given recent evidence that soluble UA itself may mediate cellular damage (30), we also evaluate the effect of an acute UA challenge on COM crystal adhesion. Exposure of cells to 0.01 or 0.1 mg/ml UA did not increase crystal binding over the next 24 h (Figure 6B). However, exposure of cells to 0.5 mg/ml UA, similar to the concentration often found in human urine, increased COM crystal adhesion 1 and 4 h later, which returned to baseline after 24 h. Therefore, we cannot exclude the possibility that partial dissolution and release of free UA could mediate the positive effect of UA crystals on COM crystal binding. Second, our experiments suggest that hyperuricosuria, independent of the formation of UA crystals, could potentiate COM crystal retention on the kidney.

**Discussion**

These studies provide evidence that adhesion of COM crystals is enhanced to proliferating, migrating renal cells. The presence of exogenous PGE₂ seems to modulate this response, which involves cell-surface expression of glycoconjugates and sialic acid residues. As evidence suggests that sialic acid–containing glycoproteins on the renal cell surface can mediate COM crystal adhesion (5), PGE₂ could exert its action by regulating expression or exposure of these molecules on the surface of regenerating cells. If cells along the distal renal tubule in vivo respond similarly to these forms of stress, then it is possible that subtle renal injury could increase the likelihood of crystal retention and eventual kidney stone formation. Therefore, intrarenal PG could serve a protective function by preventing adhesion of crystals to regenerating cells.

Human and rat studies have suggested that tubular injury
may play a role in stone formation. Increased excretion of cellular enzymes has been observed in the urine of stone-forming humans (18,19) and of oxalate-loaded rats (20), and it has been postulated that cellular damage could result from crystal deposition. However, when the nephrotoxin gentamycin was administered to rats together with oxalate, crystal deposition was enhanced (31), suggesting that cellular damage might precede and promote crystal retention, rather than be a consequence of the crystals.

Experiments in the scrape-wounding model suggest that cell-surface crystal binding proteins are present on the surface of regenerating and migrating cells (Figure 4B). Candidate molecules include hyaluronan, which has been demonstrated on the surface of migrating renal cells in association with crystals (17), and heparan sulfate proteoglycan, because the protein was immunohistochemically localized to regions of ethylene glycol–treated rat kidneys that contained crystalline deposits (32). Because proliferating cells can be susceptible to crystal binding for a period of days, PGE2 could play an especially important role by protecting cells during this critical period. Presumably, PGE2 acts in culture to remove crystal-binding molecule(s) from the cell surface or promotes synthesis of other molecules that block access of crystals to their receptors on the cells (see Figure 7). Results of our previous study with PGE2 supports the latter possibility, because the effect of PGE2 required RNA transcription, new protein synthesis, and N-glycosylation (15). Furthermore, presence of the N-glycosylation inhibitor tunicamycin blocked the decline in crystal binding from days 3 to 5 after plating (Figure 1B), as well as to proliferating, migrating cells (Figure 4D). Therefore, the bulk of evidence suggests that progressive synthesis of complex cell-surface N-glycosylated proteins of the glycocalyx protects against crystal binding, perhaps by blocking access to crystal-binding molecules (CBM); right). On balance, treatment with tunicamycin, a blocker of N-glycosylation, prevents a decline in crystal binding to cells as a mature monolayer is established, supporting the idea that side chains of the glycocalyx are protective and that the majority of sialic acid residues on terminal components of the glycocalyx are not involved in crystal binding (indicated as ◊). Nonselective removal of both classes of molecules (e.g., with trypsin) would decrease crystal binding to cells.

Synthetic D-mannosamine analogues with elongated aliphatic N-acyl groups can act as precursors of biosynthetically modified sialic acids (23). In tissue culture, as well as in vivo, these compounds are taken up by cells without apparent toxicity and are efficiently processed by the sialic acid biosynthetic pathway (23). When MDCKII cells were grown by Keppler et al. (23) with these N-substituted precursors, the incorporation of the modified sialic acid ranged between 18 and 35% of total cell sialic acids. Treatment of MDCKII cells with ManNProp decreased influenza A virus infection of the cells by 60%, whereas ManNPent pretreatment decreased it by 80% (23). In the present study, incorporation of ManNPent and ManNProp also reduced COM crystal adhesion (Figure 8). Therefore, biosynthetically introduced modifications of sialic acids and presumably cell-surface sialoglycoconjugates were sufficient to alter the cell–crystal interaction. However, the number and the type of binding interactions that influence adhesion of a COM crystal to a cell probably differ from those that mediate influenza A virus infection (23), and only a minority of cell-surface sialic acid residues are likely to have incorporated the synthetic precursor analogue (23), perhaps explaining the different magnitude of inhibition seen. Nevertheless, these results suggest that cell-surface sialic acid residues play an important role in the interaction between renal cells and calcium oxalate crystals.

Could altered cell-surface expression of hyaluronic acid (33) link the findings in our study? PGE2 has been demonstrated to
enhance hyaluronan production by various cell types (34). Glycosylation of cell surface CD44, including the presence of sialic acid residues, seems to decrease hyaluronic acid binding to cells (35). Therefore, if hyaluronan were the sole mediator of the crystal binding effects observed in this study, then one would predict that treatment with PGE2, neuraminidase, or tunicamycin might increase crystal binding to cells, whereas the opposite was observed. The effect of sialic acid precursors would be harder to predict, because it would depend on how the side chain modifications of sialic acid residues within the CD44 molecule altered its interaction with hyaluronan. It is possible that important cell-type differences in response to PGE2 could explain some of these apparent discrepancies. However, further study will be required to determine the precise interactions of PGE2, sialic acid, hyaluronan, and crystal binding.

The series of events by which freshly nucleated crystals are retained in the kidney and initiate nephrolithiasis are poorly understood. However, binding of microcrystals to the apical surface of tubular cells (5,11) or perhaps nucleation on the cell surface (36) followed by cellular processing of the crystals (11,37) could be important determinants of intranephronal calcification. Our study supports the hypothesis that injury of tubular cells favors crystal deposition (33) and possibly promotes kidney stone formation. Potential injuries could be subtle, such as transient renal ischemia. In an autopsy study of bivalved kidneys, only a history of hypertension correlated with the presence or absence of papillary calcifications, even though multiple other clinical parameters were evaluated (38). Over the years, multiple epidemiologic studies have demonstrated an association between hypertension and nephrolithiasis, although the underlying pathogenic explanation remains obscure (39–41). Our study suggests another hypothesis: That regional, perhaps transient, ischemia in the nephrosclerotic kidney could predispose tubular cells to crystal adhesion, leading to their retention and eventual kidney stone formation. Of note, in humans, acute tubular necrosis is not associated with nephrolithiasis. However, the chemical composition of the urine is different under these conditions (e.g., decreased calcium excretion), and it might not be supersaturated enough to support stone growth.

In our model system, UA crystal pre-exposure also promoted COM crystal binding for the next 24 h. Other investigators have suggested that renal cells actively respond to urate crystals. In response to monosodium urate (MSU) crystal addition, MDCK cells seemed enlarged and raised above a monolayer culture in clumps termed “reaction sites” (29). Intracellular MSU crystals were detected by electron microscopy, and release of cellular lactate dehydrogenase into the medium was demonstrated, which suggested cell injury. Increased COM crystal adhesion to cells that have “reacted” to UA crystals could in part explain the association between hyperuricosuria and calcium oxalate stone formation (28). It is interesting that
cellular uptake of COM crystals also enhanced adhesion of additional COM crystals (37). It is also of interest that cells that were exposed to soluble UA bound more crystals, especially because increasing evidence is emerging that UA can cause cellular damage and promote renal disease (30). Therefore, hyperuricosuria, with or without UA crystal formation, could promote calcium oxalate stone disease via several different pathways. Because UA or mixed UA stone formers are characterized by a lower urinary pH, whereas pure hyperuricosuric calcium oxalate stone formers are not (1), different mechanisms could predominate in different population groups. Finally, several of the mechanisms observed in the current study might also act in concert. For example, hypertensive patients with nephrolithiasis often have hyperuricosuria (42).

In summary, the COM crystal-binding capacity of proliferating renal cells, including as a response to injury, is enhanced and PGE₂ can ameliorate this response in many circumstances. Sialic acid residues within cell-surface glycoconjugates seem to be crucial for crystal binding to proliferating cells. Subtle renal tubular cell injury may be an important precursor for crystal deposition in the kidney and eventual stone formation.

Acknowledgments

This work was supported by grants to J.C.L. from the National Institutes of Health (DK 53399, DK 60707) and the Oxalosis and Hyperoxaluria Foundation.

We thank Y. Nakagawa for preparation of valuable reagents and discussions and V. Kumar and F.G. Toback for valuable advice and discussions.

References


