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ROLE OF CALCIUM OXALATE MONOHYDRATE CRYSTAL INTERACTIONS WITH RENAL EPITHELIAL CELLS IN THE PATHOGENESIS OF NEPHROLITHIASIS: A REVIEW

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Abstract

Renal tubular fluid in the distal nephron is supersaturated with calcium and oxalate ions that nucleate to form crystals of calcium oxalate monohydrate (COM), the most common crystal in renal stones. How these nascent crystals are retained in the nephron to form calculi in certain individuals is not known. Recent studies from this laboratory have demonstrated that COM crystals can bind within seconds to the apical surface of renal epithelial cells, suggesting one mechanism whereby crystals could be retained in the tubule. Adherence of crystals to cells along the nephron may be opposed by specific urinary anions such as glycosaminoglycans, uropontin, nephrocalcin, and citrate. In culture, adherent crystals are quickly internalized by renal cells, and reorganization of the cytoskeleton, alterations in gene expression, and initiation of proliferation can ensue. Each of these cellular events appears to be regulated by extracellular factors. Identification of molecules in tubular fluid and on the cell surface that determine whether a crystal-cell interaction results in retention of the crystal or its passage out of the nephron appears critical for understanding the pathogenesis of nephrolithiasis.

Key Words: Adhesion, anions, BSC-1 cells, brushite, endocytosis, gene expression, glycosaminoglycans, hydroxyapatite, interstitial fibrosis, Madin-Darby canine kidney (MDCK) cells, mitogen.

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Introduction

Renal tubular fluid in the distal nephron is supersaturated with calcium and oxalate ions that nucleate to form crystals of calcium oxalate (CaOx) monohydrate (COM), the most common crystal in renal stones. How these nascent crystals are retained in the kidney to form calculi in certain individuals is unknown. Given known rates of crystal growth and tubular fluid flow, calculations suggest that a single crystal would not become large enough to occlude the lumen during its transit through the nephron [13]. A recent study from this laboratory indicates that renal epithelial cells rapidly and specifically bind and internalize COM crystals, suggesting one mechanism whereby these crystals could be retained in the nephron [33, 35] and subsequently form a kidney stone [17, 36, 37]. Here we present experimental results that support a new hypothesis: factors which modulate the crystal-cell interaction are critical determinants in forming of a renal calculus.

COM crystals and human kidney cells

Opportunities to study intracellular crystals *in vivo* are limited in part because histological examination of kidneys from patients with nephrolithiasis is notoriously difficult, as calcifications often disrupt tissue architecture during sectioning, and calcium deposits can be lost during tissue fixation [45]. The existing evidence that crystal-renal cell interactions occur *in vivo* is partially derived from case reports of patients with disorders of oxalate metabolism. For example, deposition of CaOx crystals in tubular cells of a transplanted kidney was observed in a patient with primary hyperoxaluria days after kidney-liver transplantation in association with marked oxalate excretion [34]. In some tubular epithelial cells, crystals were seen, whereas others exhibited proliferation or multinucleated giant cell formation. Human kidney tubular cells containing CaOx crystals, in some instances undergoing proliferation, have also been described in other states of high urinary excretion of oxalate such as primary hyperoxaluria [50], malabsorption secondary to Crohn's disease and chronic pancreatitis

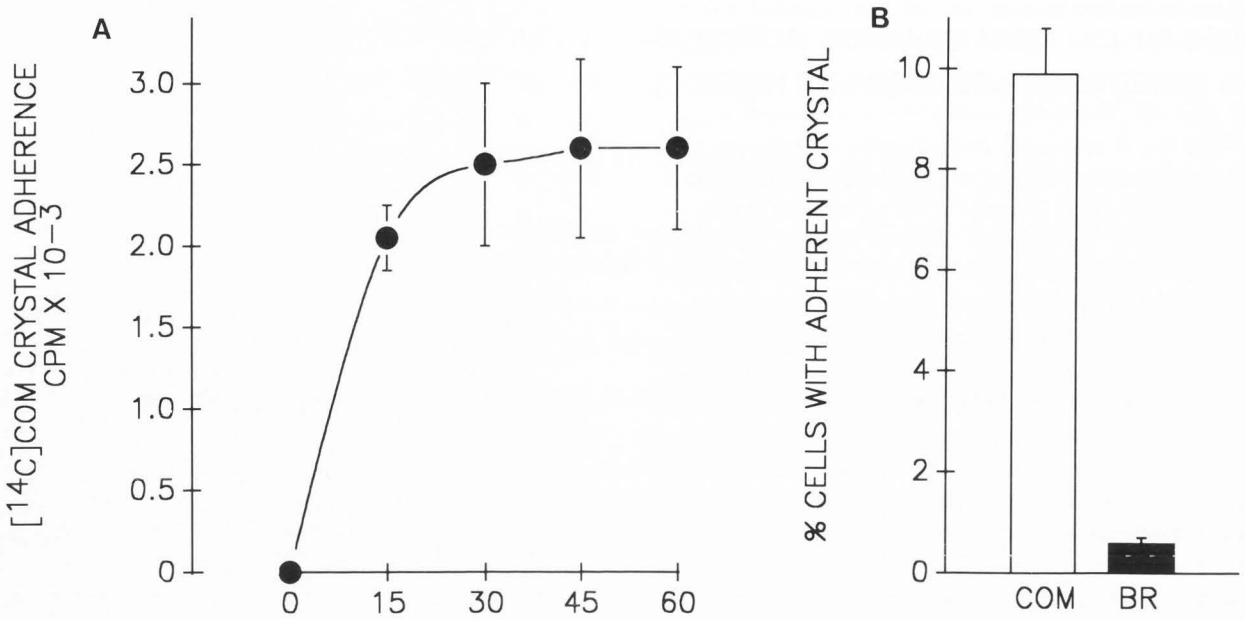

Figure 1. Kinetics and crystal-type specificity of crystal adhesion to BSC-1 cells. High-density, quiescent cultures were prepared, and COM crystals were added from a sterile suspension in water to the culture medium. **(A)** Association of [^{14}C]COM crystals with BSC-1 cells. At specified times after addition of [^{14}C]COM crystals, the monolayer was washed 3 times with phosphate-buffered saline, scraped into a scintillation vial, and radioactivity was measured. Association of crystals was greater than control after 15 seconds, and maximal by 30 seconds ($p < 0.001$). **(B)** Cells were examined under a polarizing microscope for the presence of associated particulates 2 minutes after addition to the medium. Binding of COM crystals was significantly greater than for brushite (BR). Each value is the mean {error bars are $\pm$ the standard error (SE)} for at least 3 cultures. Adapted from Lieske *et al.* [37].

[40, 63], and following intestinal bypass surgery for obesity [15]. These observations in human kidney tissue suggest that renal epithelial cells can bind and internalize CaOx crystals *in vivo*. Also, when high-density, quiescent cultures of non-transformed monkey renal epithelial cells (BSC-1 line) were used as a model of the distal tubular epithelium to study crystal-cell interactions, COM crystals were internalized by the cells and stimulated proliferation, thereby duplicating responses observed in the human kidney [33, 35].

Adhesion and internalization of crystals by renal epithelial cells

The apparent affinity of COM crystals for molecules on the apical surface of renal epithelial cells favors the binding of nascent crystals that form in supersaturated tubular fluid. Furthermore, relatively slow flow at the tubular fluid-cell interface as a result of hydrodynamic forces would promote crystal-cell interactions, especially in the predicted "dead zones" of very sluggish flow [51].

To define the time course of adhesion and internalization of a typical urinary crystal by monkey kidney epithelial cells, [^{14}C]COM crystals (300 μg/ml medium; 70.8 μg/cm^2 cell surface) were added to the medium of high-density, quiescent monolayer cultures (BSC-1 line). Adherence of crystals to these cells was detected as early as 15 seconds, and binding reached a plateau by 30 seconds (Fig. 1A). Addition of crystal amounts from 25 μg/ml (5.9 μg/cm^2) to 300 μg/ml (70.8 μg/cm^2) was associated with a linear increase in binding from 2.3 $\pm$ 0.3 to 43.2 $\pm$ 1.0 μg/10^6 cells after 2 minutes [37]. The quantity of crystals adherent to confluent canine kidney epithelial cells {Madin-Darby canine kidney (MDCK) line} and fibroblasts (Balb/c3T3 cells) also increased linearly as a function of the amount added. To confirm that cell-associated radioactivity represented adherent crystals, BSC-1 cells were examined under a light microscope two minutes after addition of crystals (200 μg/ml). Inspection revealed that 9.7 $\pm$ 0.2% of cells had adherent crystals compared to only 0.8 $\pm$ 0.2% of cells exposed to calcium phosphate crystals {brushite (BR); 200 μg/ml} two minutes after addition (Fig. 1B). Thus, COM crystals rapidly and specifically adhere to renal epithelial cells in culture, suggesting inherent recognition characteristics on the crystal and kidney cell surfaces.

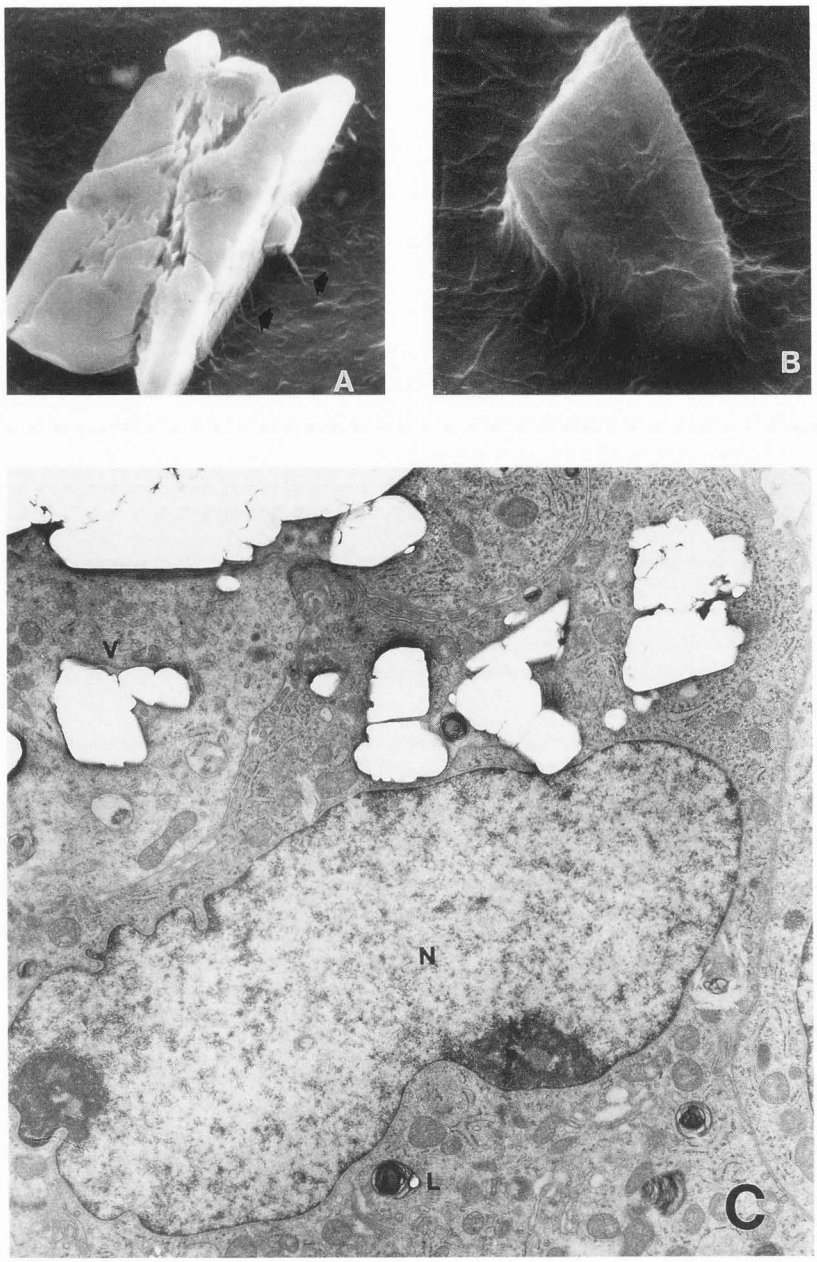

Figure 2. Scanning and transmission electron micrographs showing adhesion and internalization of COM crystals by BSC-1 cells. High density, quiescent cultures were exposed to COM crystals for specified periods of time, then fixed with half-strength Karnovsky solution. For SEM (**A** and **B**), cells were air dried, mounted on a stub, coated with gold and examined with a scanning microscope at an accelerating voltage of 40 kV. For TEM (**C**), fixed cells were dehydrated in increasing concentrations of ethanol (35% to absolute) and embedded in Epon epoxy resin. Ultrathin sections were cut on an ultramicrotome, stained for 1 hour with uranyl acetate and for 3 minutes with lead citrate, and then examined at an accelerating voltage of 80 kV with an electron microscope. (**A**) A high-magnification view of a crystal on the cell surface demonstrates multiple microvillar projections (arrows) extending over the crystal at 15 minutes; photo width (P.W.) = 17 μm. (**B**) A crystal under the plasma membrane, after engulfment (30 minutes); P.W. = 5.7 μm. (**C**) Multiple intracellular crystal aggregates lie within membrane-lined vesicles (V) at 6 hours. A lysosomal body (L) and the nucleus (N) are seen; P.W. = 10.2 μm.

The structural correlates of binding and uptake of COM crystals by BSC-1 cells was first characterized by scanning electron microscopy (SEM) in this laboratory [36]. Microvilli on the apical cell surface appeared to make the initial contact with a crystal (Fig. 2A). With time, the number of microvilli interacting with the crystal surface became more numerous. Eventually, the crystal was seen immediately under the plasma membrane (Fig. 2B). Alterations in brush border microvilli of proximal tubular epithelial cells were also noted in a rat model of oxalate nephropathy [27], providing evidence *in vivo* of the importance of these structures for the initial crystal-cell interaction.

The apparent intracellular location of COM crystals observed with the polarizing microscope [35] was confirmed by transmission electron microscopy (TEM) [33]. After 30 minutes, many BSC-1 cells contained COM crystals in a peripheral location just under the plasma membrane [33], and at one hour, crystals were often in a central location within the cell (Fig. 2C). In many instances, a membrane-like structure appeared to surround the crystal, suggesting that it was within a vesicle. These studies indicate that endocytosis can occur as early as 30 minutes after exposure of BSC-1 cells to COM crystals.

These structural and functional studies of crystal-cell

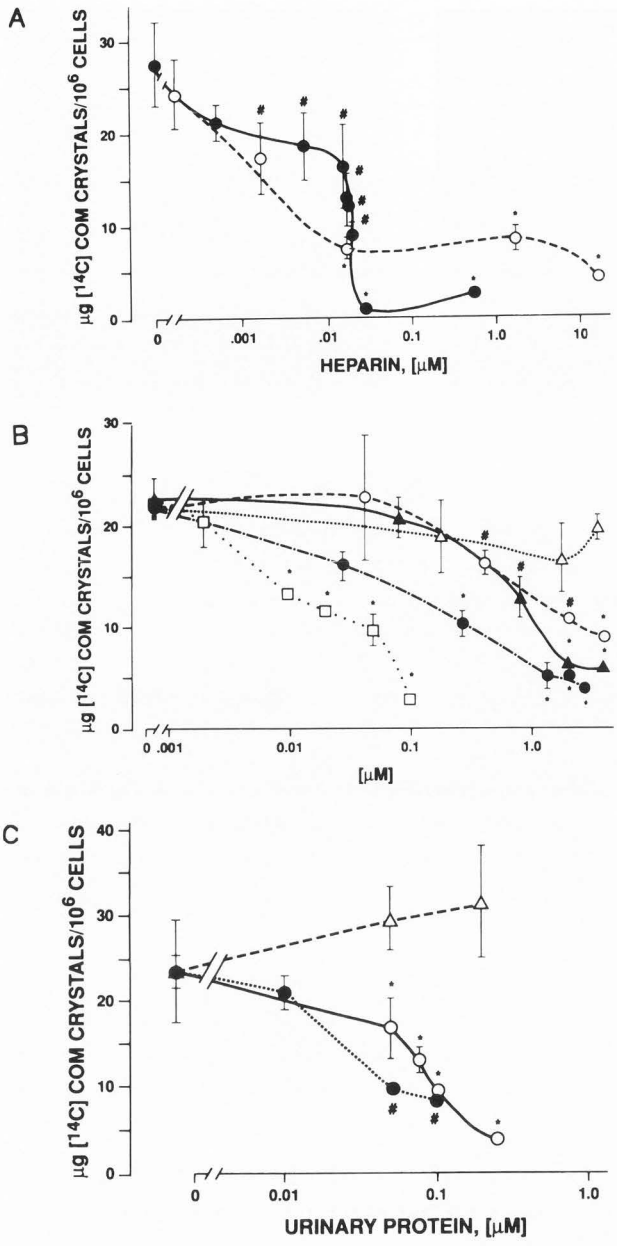

Figure 3. Inhibition of COM crystal binding to BSC-1 cells by diverse anions. High-density, quiescent cultures were prepared, the medium was aspirated and replaced with buffer containing specified concentrations of the anion under study, and [^{14}C]COM crystals (200 μg/ml) were added. Two minutes later, crystals and buffer were removed, the cell layer was rinsed three times, and cell-associated radioactivity was measured. **(A)** COM crystal binding was diminished when heparin (18 kDa, closed circles) was present at concentrations greater than 0.006 μM. Lower molecular weight heparin (6 kDa, open circles) also inhibited COM crystal adhesion when present at concentrations in excess of 0.017 μM. **(B)** The effect of diverse glycosaminoglycans on COM crystal adhesion is shown. Crystal adherence was inhibited by chondroitin sulfates A (open circles) and B (closed circles), heparan sulfate (closed triangles), and hyaluronic acid (open squares), but not by chondroitin sulfate C (open triangles). **(C)** The effect of human urinary proteins is shown. Crystal adherence was inhibited by nephrocalcin (open circles) and uropontin (closed circles), but not by THP (open triangles). $^{\#}p < 0.05$; $^{*}p < 0.001$ compared to control. Adapted from Lieske *et al.* [37].

interactions in culture indicate that COM crystals rapidly adhere to microvilli on the cell surface and are subsequently internalized. This cell behavior provides a dynamic model to explain the presence of intracellular CaOx crystals in the kidneys of patients with hyperoxaluria [15, 34, 40, 50, 63] and suggests a potential mechanism underlying the formation of kidney stones.

Adhesion of crystals to renal epithelial cells is inhibited by specific anions

We sought to identify molecules that alter the rapid adhesion of COM crystals to the surface of renal epithelial cells. Heparin (molecular weight, MW = 18 kDa), a potent inhibitor of COM crystal growth [1], maximally inhibited adherence of these crystals to cells by 95% at concentrations above 0.03 μM [37] (Fig. 3A). Heparins of lower MW (6 and 3 kDa) also inhibited COM crystal adhesion. Neither monomeric subunits of the heparin molecule nor six different heparin disaccharides were effective, suggesting that more than two repeating units of this glycosaminoglycan are required for an inhibitory effect [37]. Adhesion of COM crystals to MDCK cells and to 3T3 fibroblasts was also inhibited by heparin, demonstrating that its inhibitory effect is not specific for renal epithelial cells [37].

Although heparin is not found in urine, other sulfate-containing glycosaminoglycans are [55]. Chondroitin sulfates A and B each inhibited COM crystal adherence when present at concentrations above 1.4 μM and 0.4 μM, respectively, whereas concentrations of chondroitin sulfate C as high as 37 μM had no effect [37] (Fig. 3B). Heparan sulfate at concentrations above 0.8 μM decreased crystal adherence, as did hyaluronic acid above 0.01 μM [37] (Fig. 3B). Thus, several sulfated and non-sulfated glycosaminoglycans found in urine can block COM crystal adherence, but not all members of this class of molecules can do so.

Other extracellular anions also reduced adhesion of COM crystals to the cell surface. Polyaspartic or polyglutamic acid, anions that are potent inhibitors of COM crystal growth [21], blocked adhesion of COM crystals to cells at concentrations above 0.007 μM and 0.02 μM,

respectively [37]; the cations polyarginine and polylysine, employed as control molecules, had no effect when present in concentrations as high as 1 μM [37]. Pentosan polysulfate, a synthetic anion that strongly inhibits COM crystal growth and appears in urine when administered orally [41, 56], blocked COM crystal adherence to cells when present at concentrations above 0.33 μM [37]. Polyvinyl sulfate and dextran sulfate, which are not found in urine, also blocked crystal binding at concentrations in excess of 0.1 μM and 0.01 μM, respectively; sodium sulfate was ineffective. The polyanion citrate blocked adherence of COM crystals when present at concentrations above 250 μM [37], a level similar to that ordinarily found in human urine [43]. Therefore, polysulfated and non-sulfated polyanionic molecules can inhibit binding of COM crystals to renal epithelial cells.

Several urinary proteins that appear to play a role in nephrolithiasis have been identified. Nephrocalcin and uropontin are each potent inhibitors of COM crystal growth [54, 65], whereas Tamm-Horsfall protein (THP) inhibits aggregation of these crystals [19]. Nephrocalcin inhibited COM crystal adhesion to cells by 28% at a concentration of 0.025 μM, with maximal inhibition of 84% at 0.25 μM, whereas uropontin blocked COM crystal binding by 73% at a concentration of 0.1 μM [37]. THP had no effect on crystal adhesion [37] (Fig. 3C). The concentration range of each protein studied was similar to that found in human urine [32, 42, 54].

To determine if polyanions that blocked crystal adhesion exerted their effect on the surface of crystals and cells, or both experiments were designed to coat cells or crystals separately with the active compounds. Adhesion of crystals previously coated with heparin, chondroitin sulfate B, dextran sulfate, polyaspartate, polyglutamate, nephrocalcin or uropontin was in each case lower than the binding of uncoated control crystals [37]. When coated with polyarginine, polylysine or albumin, crystals bound to cells to the same extent as did uncoated crystals [37]. Binding of uncoated COM crystals to cells that had first been exposed to the molecules under study did not differ from adhesion of crystals to the uncoated monolayer [37]. Thus, the capacity of specific polyanions to inhibit adhesion of COM crystals to BSC-1 cells appears to be mediated by their ability to act on the crystalline surface.

The relative inhibitory capacity of each of the anions of interest was evaluated by using Langmuir-type isotherm analysis in which the reciprocal of the percent maximal binding is plotted against the inverse of the inhibitor concentration [37]. For each of the anionic inhibitors, the Langmuir isotherm plot was linear. This is consistent with the interpretation that inhibitory molecules bind to a single class of sites on the crystal surface that is crucial for adhesion to the plasma membrane, and that once these sites on the crystal are coated with inhibitory molecules, adhesion to the cell is blocked [1, 37]. Based on their calculated affinity constants, hyaluronic acid, dextran sulfate, heparin, polyaspartic acid, polyglutamic acid, polyvinyl sulfate, uropontin and nephrocalcin have the greatest affinities for the presumed binding sites on the crystal surface that mediate adhesion to cells, with values ranging from 0.013 μM (hyaluronic acid and dextran sulfate) to 0.107 μM (nephrocalcin) [37].

Crystal-cell interactions are also modified by polyanions in other experimental systems. In a study of crystal-induced lysis of red blood cells, the crystal growth inhibitors citrate and pyrophosphate were shown to decrease attachment of COM, hydroxyapatite (HA) and monosodium urate crystals to the erythrocyte membrane [39]. Cellular factors may influence crystal binding as well. The presence of specific cell-surface binding sites for COM crystals on rat medullary cells has been proposed [46]. In primary cultures of these cells, binding of COM crystals was a saturable process partially antagonized by HA crystals. Preferential binding of COM crystals to areas of the culture monolayer in which cells had spontaneously rounded up prior to addition of crystals was observed, and cells that avidly bound crystals expressed basolateral surface antigens on their apical surfaces [47]. Additional evidence for the existence of specific plasma membrane crystal-binding sites was obtained when cells were pretreated with ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) to expose basolateral epitopes which also increased binding of COM crystals [47]. These experiments, and the observation that COM crystals adhere to injured regions of rat bladder epithelium [28], suggest that crystal-binding sites may be minimally exposed under physiological circumstances but are unmasked when cells are injured or possibly during repair after injury [47]. The identity of the specific crystal-binding molecules on the renal cell surface remains undefined, although it has been suggested that membrane phospholipids may play a role [38].

Regulation of crystal endocytosis by renal epithelial cells

Molecules have been identified that modulate internalization of COM crystals by BSC-1 cells. Epidermal growth factor (EGF) and the nucleotide adenosine diphosphate (ADP) [20, 22, 61], mitogens that also stimulate migration of these cells in scrape-wounded monolayer cultures [23], each increased COM crystal uptake by 55% and 52%, respectively [33] (Fig. 4A). Two other mitogenic signals, raising the calf serum concentration or exposing cells to low-potassium (low-K) medium [24] each augmented uptake of crystals (Fig. 4A). Thus, diverse signals that alter cellular function and are

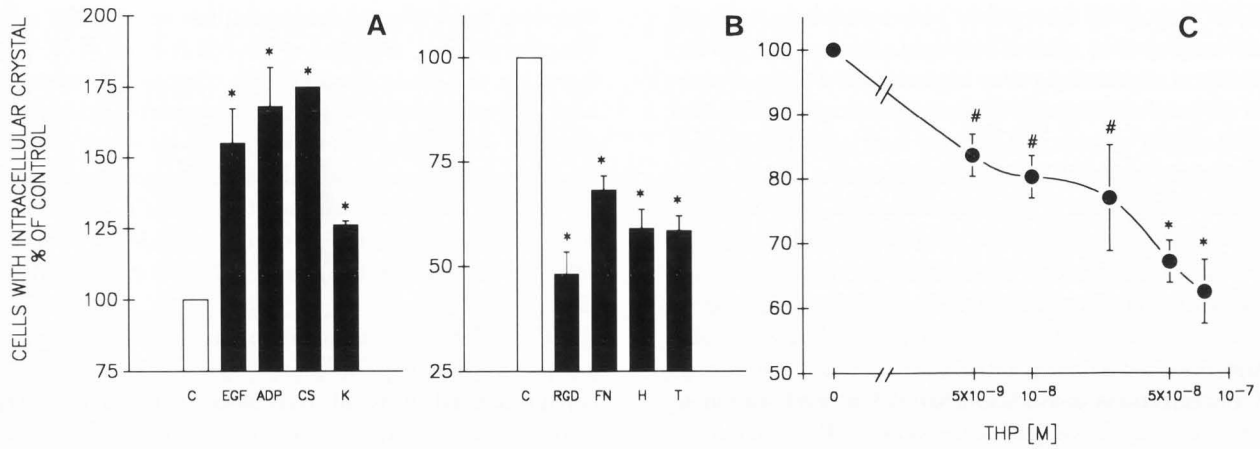

Figure 4. Positive and negative regulation of COM crystal endocytosis. High-density, quiescent cultures of BSC-1 cells were prepared, and the agent of interest was added to the medium. To assess the effect of reduced K concentration, spent medium was aspirated and replaced with fresh Dulbecco's modified Eagle's medium (DMEM) containing 0.5% serum, 1.6 μM biotin and a K concentration of 3.2 mM (low-K) or 5.4 mM (control, C). Fifteen minutes later, a sterile suspension of COM crystals in water was added to achieve a concentration of 100 μg/ml. One hour afterwards, the medium was aspirated, the monolayer was trypsinized, and cells were assayed for the presence of internalized crystals. TEM confirmed that cell-associated crystals viewed under polarized microscopy were within the cell [33]. Trypsinization did not visibly alter cell-associated crystals, although dislodgement of partially internalized crystals cannot be excluded. (**A**) COM crystal uptake was enhanced 58% by epidermal growth factor (EGF, 50 ng/ml), 68% by adenosine diphosphate (ADP, 20 μM), 75% by calf serum (CS, 1%), and 26% by low-K medium (K). (**B**) Crystal uptake was decreased 52% by the tetrapeptide RGDS (RGD, 50 μg/ml), 32% by fibronectin (FN, 5 μg/ml), 41% by heparin (H, 10 μg/ml) and 41% by TGF-β2 (T, 2 ng/ml). (**C**) Tamm-Horsfall glycoprotein (THP) purified from normal human urine inhibited COM crystal endocytosis at concentrations $\geq 5 \times 10^{-9}$ M. Each value is the mean ($\pm$ SE) for at least 4 cultures. $^{\#}p < 0.01$; $^{*}p < 0.001$. Adapted from Lieske and Toback [33].

mitogenic for BSC-1 cells stimulate endocytosis of COM crystals, although the pathways by which they do so likely differ from each other. Two of these signals are known to modify cell structure: cytoskeletal changes occur after exposure to ADP [24], and alterations in the plasma membrane and microvilli take place after exposure to low-K medium [60]. These changes in cell structure may contribute to enhanced uptake of crystals.

Negative regulators of COM crystal uptake were identified by testing the hypothesis that agents which retard cell migration might inhibit endocytosis. Heparin, transforming growth factor-β2 (TGF-β2), and the tetrapeptide arginine-glycine-aspartic acid-serine (RGDS) each impede migration of BSC-1 cells in wounded monolayer cultures [23], the latter apparently by binding to a cell-surface integrin receptor [48]. These three factors decreased COM crystal uptake by 36%-41% [33] (Fig. 4B). The extracellular protein fibronectin which contains an RGD sequence inhibited COM crystal uptake by 33% (Fig. 4B), suggesting that RGDS might exert its effect by binding to a fibronectin receptor on the cell surface. Heparin exerts disparate effects on cell function, including inhibition of growth and extracellular matrix (ECM) protein synthesis in smooth muscle cells [64]; blocking induction of early-response genes by agents that activate protein kinase C [44]; uncoupling of the α-adrenergic receptor from its G protein in rat liver [8]; and inhibiting renal epithelial cell migration [23]. Thus, the mechanism(s) by which heparin inhibits COM crystal uptake is unclear. How TGF-β2 exerts its inhibitory effect on endocytosis is also unknown, but since this negative autocrine growth factor is present in cells of the collecting duct [12] and distal tubule [58], it is tempting to speculate that the protein, which is also found in urine, might prevent endocytosis of crystals by epithelial cells lining the urinary tract under physiological conditions.

The effect of THP on crystal endocytosis was investigated because it is the most abundant protein in human urine [32], although its role in renal physiology remains uncertain. The glycoprotein is synthesized in the thick ascending limb of Henle's loop (TALH) and early distal tubule where it is anchored to cell-surface lipids by a phospholipase C-sensitive linkage from which it is cleaved and released into tubular fluid [32]. THP is a potent inhibitor of COM crystal aggregation *in vitro* [18] and binds to specific cytokines such as tumor necrosis factor and interleukin-1 [52, 53]. It has been proposed that THP defends cells of the nephron against bacterial

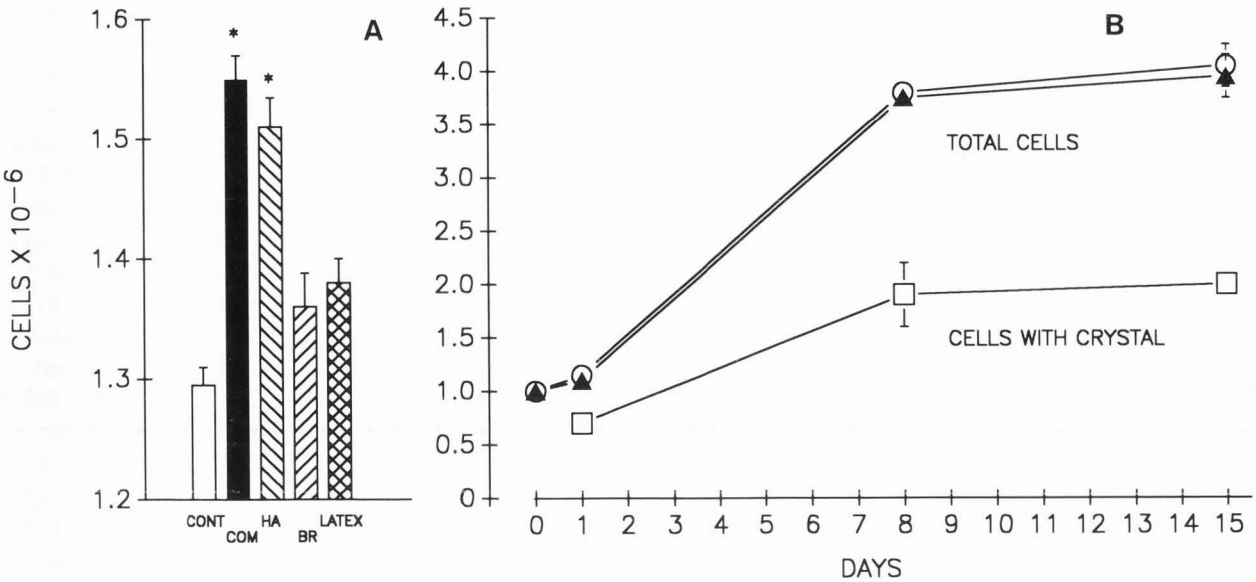

Figure 5. Long-term effects of urinary crystals on growth of BSC-1 cells. Subconfluent cultures of BSC-1 cells were prepared. (A) COM, hydroxyapatite (HA) or brushite (BR) crystals or latex beads were added to the culture medium to achieve a final concentration of 10 μg/ml. The number of cells was counted 4 days later in a hemocytometer. Multiplication was stimulated 19% by addition of COM crystals and 17% by HA crystals ($p < 0.001$); cont = control. (B) COM crystals (50 μg/ml) were added to BSC-1 cells on day 0. Non-adherent crystals were removed by a fluid change on day 1. One and 2 weeks later, the number of cells per culture and the presence of intracellular crystals was assessed. The total number of cells in cultures exposed to COM crystals did not differ from the control (open circles) 7 or 14 days after exposure. The number of cells containing intracellular crystals increased from 0.7 X 10^6 on day 1 to 1.8 X 10^6 on day 7 (open squares). Each value is the mean $\pm$ SE for 6 cultures. * $p < 0.001$ compared with control. Adapted from Liekse *et al.* [35, 36].

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adhesion and colonization, and maintains water impermeability of the TALH [32]. THP isolated from human urine decreased COM crystal endocytosis by 34% at a 10-fold lower concentration (5 x 10^{-8} M) than is found in normal urine [18] (Fig. 4C). THP, as well as fibronectin and heparin, appear to inhibit endocytosis of COM crystals by acting on the cells, not by coating the crystals, because crystal internalization was reduced by 27-37% when cells but not crystals were pre-incubated with each agent. Thus, the capacity of THP in distal tubular fluid to block uptake of COM crystals may play a role in preventing renal stone formation.

Endocytosis of crystals is associated with changes in specific components of the cytoskeleton. For example, actin filaments are necessary for phagocytosis by macrophages, and in these cells uptake of particulates is blocked by cytochalasin B [2]. Phalloidin, which selectively binds to F (filamentous)-actin but not to G (globular)-actin, stained a domain of BSC-1 cells just beneath adherent COM crystals one to three hours after their addition to the medium [36]. The staining appeared to follow the crystal profile during its engulfment, suggesting a role for actin during internalization.

In summary, internalization of COM crystals by renal epithelial cells can be positively and negatively regulated by diverse factors, some of which are constituents of normal urine. The impact of these factors on crystal-cell interactions could determine whether the crystal is retained in the nephron or passes out of it in tubular fluid.

Internalization of crystals and cell proliferation

Multinucleated giant cell formation and proliferation were prominent features in a human kidney 16 days after it was transplanted into a patient with primary hyperoxaluria [34]. This observation prompted us to test the hypothesis that COM crystals are mitogenic for renal epithelial cells. We found that multiplication of BSC-1 cells increased progressively as the concentration of COM crystals was raised to a maximum of 10 μg/ml (Fig. 5A). When added to high-density, quiescent cultures of these cells, the crystals stimulated [^{3}H]thymidine incorporation into DNA up to 3.5-fold. Importantly, the kinetic profile of stimulated DNA synthesis differed between COM crystals and another mitogenic signal, 10% calf serum. Maximal stimulation of [^{3}H]thymidine incorporation by serum occurred after 16-21 hours, while

enhancement by COM crystals was delayed until 24-29 hours. This eight-hour difference might represent the time required for events that transpire between adherence and endocytosis of the crystal, and the subsequent generation of a signal that initiates cell proliferation. Crystal- and cell-type specificity were observed. HA crystals stimulated growth of BSC-1 cells, whereas BR crystals and latex beads (used as a control particulate) did not (Fig. 5A) [35]. COM crystals also stimulated growth of MDCK cells, but HA and BR crystals did not. On the other hand, COM crystals were not mitogenic for Balb/c3T3 fibroblasts, whereas HA crystals were [6, 35]. As COM crystals stimulated growth of renal epithelial cells of both monkey and canine origin, but not fibroblasts, the mitogenic effect of this crystal appears to be relatively cell-type specific.

The duration of a crystal-cell interaction required to stimulate mitogenesis is relatively brief. DNA synthesis was not initiated if cells were exposed to crystals for 15 minutes and the crystals were then removed, whereas exposure for 30 minutes stimulated an increment in [^{3}H]thymidine incorporation equivalent to that observed after 24 hours [35]. Thus, adherence of a sufficient amount of crystals to the plasma membrane for $>$ 15 minutes but $\leq$ 30 minutes, and/or engulfment of a crystal may be sufficient to signal mitogenesis.

Other cellular functions appear to change after uptake of crystals. Previous studies revealed that certain mitogens for BSC-1 cells initiated surprisingly rapid alterations in a cytokeratin component of the intermediate filament network [24]. Utilizing a monoclonal antibody to cytokeratin 8 in control BSC-1 cells, staining was observed most intensely in a perinuclear zone [36]. After 8 hours of exposure to COM crystals, cytokeratin 8-containing intermediate filaments appeared to redistribute throughout the cytoplasm [36]. Furthermore, cytokeratin 8 reorganization was observed in all cells after addition of COM crystals, i.e., it was not confined to cells that internalized a crystal. Conversely, staining of cells exposed to COM crystals with a monoclonal antibody to tubulin did not reveal any morphologic alterations. Therefore, exposure of cells to COM crystals is associated with alterations of two specific components of the cytoskeleton: cytokeratin 8-containing intermediate filaments and actin. However, while actin may participate in crystal uptake, changes in cytokeratin 8 appear to occur later and could be a consequence of this process. Furthermore, as all cells in the monolayer exhibited reorganization of cytokeratin 8-containing filaments after COM crystal exposure [36], cell-to-cell communication as a response to the crystal-cell interaction is suggested, possibly mediated by a secreted autocrine factor.

At higher concentrations, CaOx crystals may also be toxic for renal epithelial cells. When 500 μg/ml of COM crystals were added to MDCK cells, some degree of cell detachment and release of both intracellular and brush border enzymes were detected (lactate dehydrogenase, N-acetyl-β-glucosaminidase, leucine aminopeptidase, and τ-glutamyl transpeptidase) [16]. Similar structural and functional changes were observed in MDCK cells exposed to oxalate ions at concentrations similar to those found in urine (200 μM) [16]. However, when another group of investigators added a lower quantity of COM crystals (32 μg/cm^2) to cultured MDCK cells, no evidence of cellular damage was observed: release of brush border (τ-glutamyl transpeptidase) and cellular (lactate dehydrogenase) enzymes were not increased, trypan blue and mannitol exclusion was preserved, and mitochondrial metabolism was unaltered [59]. Cell injury and death have been observed in rats made hyperoxaluric by oral administration of ethylene glycol [25]. Oxalate alone has also been observed to promote cell growth at lower concentrations (80-320 μM), as assessed by measurement of cell number and [^{3}H]thymidine incorporation into DNA, as well as cell death at higher concentrations (400-1600 μM) [31]. Together, these studies suggest that the cellular response to CaOx crystals or oxalate ions may be strongly concentration-dependent. It is also possible that some of the secondary cellular responses of renal epithelial cells following uptake of COM crystals may be mediated by oxalate ions subsequently released from the internalized crystal.

Long-term exposure to crystals

To determine the fate of a COM crystal within a renal cell over a prolonged period, cell viability and function were studied for two weeks after exposure to crystals (Fig. 5B). During this period, internalized crystals did not adversely affect the growth of BSC-1 cells. Furthermore, the number of cells in the culture containing one or more crystals increased between 1 and 7 days, despite a fluid change on day 2 to remove any non-adherent crystals, demonstrating that internalized crystals were allocated to daughter cells during division. Electron microscopy has also been used to study internalization of COM crystals by cultured MDCK cells, as well as possible dissolution of internalized crystals [59].

In summary, COM crystals rapidly adhere to the apical surface of renal epithelial cells, undergo internalization and can subsequently stimulate proliferation. The internalized crystals appear to reside in vesicles, need not adversely affect cell growth and can be distributed to dividing daughter cells. Thus, renal epithelial cells in culture may possess the necessary metabolic machinery to process COM crystals without apparent toxic effects at low crystal concentrations.

Gene expression after uptake of COM crystals

Identification of specific genes expressed by renal

Table 1. Kinetics of gene expression after exposure of BSC-1 cells to COM crystals.

	0 minutes	15 minutes	1 hour	2 hours	3 hours	6 hours	12 hours	24 hours
Immediate-early genes								
c-*myc*		*	**					
EGR-1			**	**	*			
Nur-77				**				
c-*jun*				**				
Extracellular matrix genes								
PAI-1	*	*	*	***	***	**	*	*
u-PA	*	*	*	*	*	*	*	*
PDGF-A chain	*	*	*	**	**	**	*	*
Connective tissue growth factor			**	*	*	*	*	*

Abbreviations: PAI-I: plasminogen-activator inhibitor-1; PDGF: platelet-derived growth factor; u-PA: urinary-type plasminogen activator. The number of asterisks (0 to 3) correspond to the relative hybridization intensity of the [^{32}P]cDNA probe used at each time. The asterisks present at 0 minutes indicate constitutive gene expression.

cells in response to COM crystals could provide fresh insight into mechanisms that mediate pathologic changes associated with hyperoxaluria and formation of kidney stones. Thus, RNA was extracted from high-density, quiescent BSC-1 cells after exposure to COM crystals (200 μg/ml) and studied by Northern analysis [17]. Immediate-early genes were examined first since expression of these genes is low or undetectable in quiescent cells but can be rapidly and transiently induced within minutes without new protein synthesis. The early-growth-response (EGR)-1 transcript was not detected under control conditions but was maximally expressed 1 to 2 hours after addition of crystals to the culture medium (Table 1). The DNA-binding protein, Nur-77, was transiently induced 2 hours after cells were exposed to COM crystals. Expression of c-*jun*, a protooncogene that functions as an immediate-early gene, was enhanced 1 to 2 hours after addition of crystals; c-*fos*, however, was not expressed under control conditions or after addition of crystals, suggesting that a JUN-JUN homodimeric transcription factor and not a JUN-FOS heterodimer forms in the nuclei of cells following exposure to crystals. Another protooncogene that functions as a transcription factor, c-*myc*, was induced in BSC-1 cells as early as 15 minutes after exposure to crystals, with maximal expression at 1 hour. As preliminary studies showed that expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin did not change when cells were exposed to crystals, probes for these genes were used to demonstrate equal loading of RNA. Therefore, COM crystals induce expression of specific immediate-early genes (Table 1).

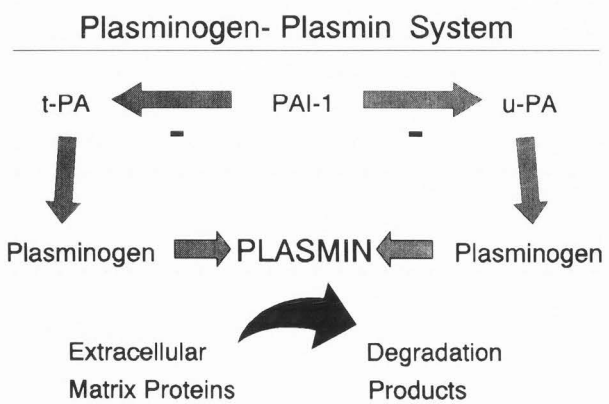

Figure 6. Regulation of plasmin production by plasminogen activator inhibitor-1 (PAI-1), urinary-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). See text for details.

Interstitial fibrosis was a prominent finding in the renal biopsy of the patient with primary hyperoxaluria we described [34], and the association of interstitial crystals, fibrosis and renal failure has been reported in many other patients with primary or secondary hyperoxaluria [4, 5, 7, 9, 10, 11, 14, 15, 30, 40, 49, 50, 63]. Therefore, in addition to the immediate-early genes, several genes that contribute to the formation and degradation of ECM via the plasminogen-activating system were studied, as new knowledge about them could provide

insight into the pathogenesis of renal scarring in this condition.

Plasmin is an extracellular broad-spectrum serine protease activated when its precursor, plasminogen, is cleaved (Fig. 6). Plasminogen is the target of two other highly specific serine proteases, urinary-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA); u-PA is primarily responsible for plasmin generation in processes involving degradation of ECM and basement membranes, whereas t-PA appears to mediate plasmin generation during fibrinolysis. Fast-acting plasminogen activator inhibitor-1 (PAI-1) is also an important component of this system. PAI-1 regulates plasmin levels by blocking the action of both the tissue- and urinary-types of plasminogen activator which in turn decreases the formation of plasmin. Reduced plasmin production could thereby result in accumulation of ECM proteins. The constitutive expression of the gene encoding PAI-1 was dramatically increased 2 to 6 hours after exposure to crystals. In contrast, constitutive expression of the gene encoding u-PA did not change (Table 1), and t-PA was not expressed by these cells. Increased PAI-1 expression without a change in u-PA could result in decreased generation of plasmin (Fig. 6). Accumulation of ECM proteins and eventual renal interstitial fibrosis would be predicted results.

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesodermal origin such as fibroblasts. It also stimulates connective tissue-forming cells to synthesize and release collagen, proteoglycans and elastic fiber proteins and acts as a chemoattractant for fibroblasts and monocytes. Increased availability of PDGF would therefore favor fibrosis. PDGF-A chain mRNA was constitutively expressed in BSC-1 cells under control conditions and was maximally stimulated 2 to 6 hours after exposure to crystals (Table 1). The gene encoding PDGF-B chain was constitutively expressed but not stimulated by crystals. Therefore, exposure to crystals enhances expression of PDGF-A chain mRNA and could increase production of this paracrine growth factor which is known to stimulate proliferation of renal fibroblasts and augment collagen production. Connective tissue growth factor (CTGF) is a cysteine-rich protein that exhibits PDGF-like biological and immunological activities [3]. Its transcript was not detected under control conditions but was induced after 1 hour of exposure to crystals and expressed continuously thereafter for up to 24 hours.

To evaluate whether particulates other than COM crystals alter gene expression, BSC-1 cells were exposed to two additional calcium-containing urinary crystals, BR and HA, as well as to latex beads. After cells were exposed to each particulate, Northern analysis revealed that only COM crystals stimulated expression of PAI-1 and PDGF-A chain mRNA. The cell-type specificity of crystal-induced gene expression was also evaluated. In canine renal epithelial cells of the MDCK line, enhanced expression of the genes encoding EGR-1 and PAI-1 was observed after exposure to COM crystals, whereas expression of neither gene was enhanced in 3T3 fibroblasts. Therefore, the capacity of COM crystals to stimulate gene expression appears to be crystal- and kidney cell-type specific.

Table 2. Unaltered expression of genes after exposure of BSC-1 cells to COM crystals.

Constitutive expression not altered	Not expressed or induced
Laminin	Stromelysin
Collagen	Collagenase
Fibronectin	IL-1α, IL-1β, IL-6
TGF-β1, TGF-β2	*gro*
Heat shock protein-70	t-PA
	bFGF
	aFGF

Abbreviations: aFGF: acidic fibroblast growth factor; bFGF: basic fibroblast growth factor; IL: interleukin; TGF: transforming growth factor; t-PA: tissue-type plasminogen activator.

The expression of other genes that regulate cell adhesion and ECM production was sought in BSC-1 cells after exposure to COM crystals. Many genes were constitutively expressed under control conditions but not altered by the crystal-cell interaction. These included laminin, collagen, fibronectin and TGF-β1 and -β2 (Table 2). The genes encoding two proteases that can degrade ECM components, collagenase and stromolysin, were not expressed in BSC-1 cells under control conditions. The gene encoding heat shock protein (HSP)-70, an intracellular protein produced in response to various forms of stress, was also studied. It was constitutively expressed in BSC-1 cells but unaltered by addition of crystals. This result supports the previous observation that COM crystals need not damage renal epithelial cells. Addition of crystals to synovial lining cells and monocytes has been reported to induce expression of genes that encode cytokines. However, the genes encoding interleukin (IL)-1α, IL-1β, IL-2, IL-6 and *gro* were not expressed in BSC-1 cells under control conditions nor after addition of crystals. The genes encoding acidic or basic fibroblast growth factor, which are important regulators of the ECM, were also not induced by

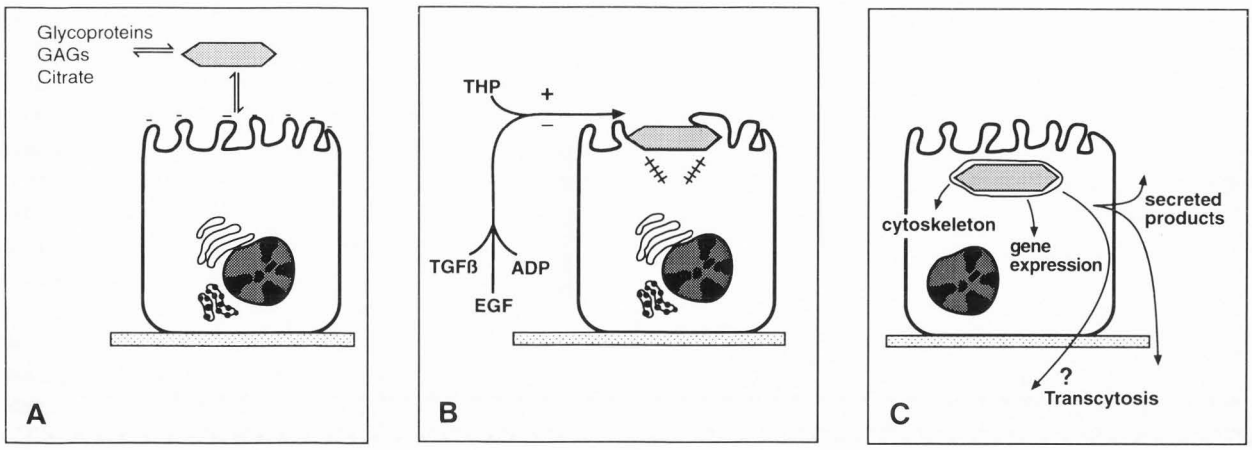

Figure 7. Sequential events {adhesion (**A**), to internalization (**B**), to cellular response (**C**)} describing the interaction between COM crystals and renal epithelial cells that may result in nephrolithiasis. GAGs = glycosaminoglycans; THP = Tamm-Horfall glycoprotein; TGF = transforming growth factor; EGF = epidermal growth factor; ADP = adenosine diphosphate. See text for discussion.

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exposure to crystals. Therefore, of the 12 genes studied that are known determinants of ECM composition, expression of only two were altered after exposure to crystals. These results suggest that stimulated gene expression after the crystal-cell interaction exposure is highly targeted within the genome.

In summary, the response of renal epithelial cells to COM crystals is characterized by increased expression of specific genes that encode: transcriptional activators (EGR-1, Nur-77, c-*jun*, c-*myc*), a regulator of the ECM composition (PAI-1) and growth factors that could stimulate fibroblast proliferation in a paracrine manner (PDGF-A chain, CTGF). Interstitial scarring observed in kidneys of patients with primary or secondary hyperoxaluria could be mediated by an interaction between CaOx crystals that form in the nephron lumen and epithelial cells of the tubular wall, with enhanced expression of specific genes whose protein products result in accumulation of ECM proteins and subsequent fibrosis. It is possible that similar changes in gene expression occur when CaOx crystalluria is present to a lesser extent, as in patients with CaOx nephrolithiasis.

Discussion

Crystal retention in the kidney could result from a specific interaction between urinary crystals and tubular cells. Renal epithelial cells do not perceive COM crystals as inert but respond to them by displaying a program of specific events that include crystal binding to the cell surface and endocytosis, altered gene expression, cytoskeletal alterations and cell multiplication [17, 33, 35, 36, 37]. In addition, cells in human renal tissue *in vivo*, and monkey and canine kidney cells in culture, exhibit similar responses to CaOx crystals [34, 35].

The encounter between a crystal formed in the nephron lumen and a tubular epithelial cell appears to be initiated by adhesion of the crystal to the apical cell surface (Fig. 7). Specific sites on the COM crystal surface that can be blocked by tubular fluid polyanions such as glycosaminoglycans and glycoproteins [37], seem to mediate binding of crystal to cell. It is also possible that specific sites such as an integrin fibronectin receptor on the cell surface [33], perhaps located on apical microvilli [36], mediate COM crystal binding. A series of cellular responses follows crystal attachment including its internalization within a vesicle, cytoskeletal rearrangements, altered gene expression, cellular proliferation and release of extracellular factors (Fig. 7) [17, 35, 36]. Additional studies will be required to delineate further this cascade of cellular responses to COM crystals and confirm a role for these processes in the pathogenesis of stone formation in humans.

Each step in the cellular response to COM crystals appears to be under a distinct set of controls (Table 3). Adhesion of COM crystals is inhibited by a diverse set of molecules present in tubular fluid including glycosaminoglycans (chondroitin sulfates A and B; heparan sulfate), glycoproteins (uropontin, nephrocalcin) and small polyanions (citrate). Internalization of crystals after adhesion also appears to be modified by distinct signals, both positively (EGF, ADP, low extracellular potassium) and negatively (THP, fibronectin, RGDS, TGF-β2) (Table 3). That THP inhibits engulfment of COM crystals suggests a specific role for this ubiquitous and puzzling urinary glycoprotein in the pathogenesis of nephrolithiasis. Finally, nephrocalcin appears to block COM crystal-induced mitogenesis [35]. It is likely that

Table 3. Factors that modify adhesion and endocytosis of COM crystals by BSC-1 cells.

	Adhesion	Endocytosis
Inhibitors	Glycosaminoglycans	Heparin
	Uropontin	Fibronectin
	Nephrocalcin	RGDS
	Polyaspartate	TGF-β2
	Polyglutamate	THP
	Citrate	
	Phosphocitrate	
	Dextran sulfate	
	DNA	
	Pentosan polysulfate	
Stimulators		EGF
		ADP
		calf serum
		low-potassium medium

Abbreviations: ADP: adenosine diphosphate; EGF: epidermal growth factor; RGDS: arginine-glycine-aspartate-serine peptide; TGF: transforming growth factor; THP: Tamm-Horsfall glycoprotein.

additional regulators of urinary crystal adhesion and/or endocytosis will be identified in the future.

The relative importance of diverse potential pathogenetic mechanisms in the formation of kidney stones remains to be determined. In pathological states associated with nephrolithiasis, it is possible that the factors which prevent crystal adhesion and internalization by cells have become ineffective and permit crystal retention by cells in the distal nephron, thereby providing an anchored nidus for subsequent crystal growth and aggregation that results in stone formation. The events that ensue after a cell binds a crystal could also contribute to stone formation. Alternatively, internalization of COM crystals could be viewed as a separate defense against stone formation. Once inside a cell, and no longer exposed to supersaturated tubular fluid, growth of a crystal would cease and its aggregation would be prevented. As an interaction with COM crystals can stimulate proliferation of renal epithelial cells [35], and mitosis reduces adhesion of cells to their ECM [57], thereby exposing previously masked plasma membrane basolateral receptors to tubular fluid, this cell response could permit adhesion of additional crystals [47]. Detachment of a crystal-laden cell could defend the kidney against crystal retention by eliminating crystals from the nephron. After cell detachment, however, the exposed tubular basement membrane might serve as a site for binding and aggregation of additional crystals, as has been demonstrated in denuded bladder epithelium [27]. Furthermore, renal epithelial cell injury and death have been observed in rat models of acute oxalate toxicity [26], so that direct injury to cells by urinary crystals and oxalate ions might facilitate binding of crystals to damaged plasma membrane domains [16, 46] or result in cell detachment and denudation of tubular basement membrane. Experimentally-induced exposure of basolateral antigens in cultured rat medullary collecting duct cells is associated with increased binding of COM crystals [47], and injury to rat uroepithelium also increases adhesion of crystals to this surface of the cell [28]. Crystal binding and uptake is associated with cellular release of proteolytic enzymes, as described after integrin activation in monocytes [62], and in this way, could mediate cell detachment followed by crystal adhesion to and aggregation upon exposed tubular basement membrane. Finally, plasma membrane fragments released from injured cells into the tubular lumen could serve as crystal nucleation and aggregation sites [29]. Further experiments are necessary to define how these diverse pathogenetic pathways might converge to retain urinary crystals in the kidney and thereby initiate the formation of calculi.

Endocytosis of urinary crystals does not appear to kill kidney epithelial cells in culture [35], and an engulfed crystal might be extruded by the tubular cell into the renal interstitium *in vivo*. Thus, exocytosis of an engulfed crystal could explain the long-known but ill-understood mechanism by which a urinary crystal finds its way into the interstitium. Since certain crystals stimulate proliferation of fibroblasts in culture (e.g., HA) [6], it is tempting to speculate that these translocated urinary crystals could initiate renal interstitial fibroblast proliferation and contribute to subsequent scarring in patients with nephrolithiasis.

In summary, crystals that nucleate in the nephron lumen may be retained in the kidney to form stones by binding to the apical surface of tubular cells and subsequently undergoing endocytosis. This formulation suggests that factors in tubular fluid that regulate the crystal-cell interaction could be critical determinants of nephrolithiasis and deserve further study.

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Discussion with Reviewers

C.F. Verkoelen: Do the authors believe that renal stones are formed from crystals that become attached to the epithelium and grow into a stone at the location of initial attachment or that renal stones are formed from crystals that, after initial attachment, have left the urinary tract by endocytosis to reappear at another location in the urinary tract?

J.G. Kleinman: On the basis of their own data or that of others, do the authors feel that the process of attachment or the process of endocytosis is more relevant to the pathophysiology of nephrolithiasis?

Authors: Our experimental results are consistent with both possibilities suggested by Dr. Verkoelen. Further study will be necessary to delineate the possible sequence of events that occur after crystal adhesion to the epithelial cell surface and the relative role of each event in nephrolithiasis. For example, the ultimate fate of an internalized CaOx crystal remains to be determined. If the cell can effectively metabolize the crystal, then crystal endocytosis could be viewed as a defense against stone formation. If crystal dissolution is a defense against stone formation, then the rate of internalization could be an important variable determining whether or not the crystal is removed from the tubular fluid before additional crystal growth and/or aggregation with other crystals can occur. The relative supersaturation of tubular fluid at the time of crystal adhesion, as well as the relative quantity of additional crystals in tubular fluid, could strongly influence the ultimate fate of adherent crystals. The impact of internalized CaOx crystals on cellular function when present for a prolonged period of time (weeks to months) are unknown, and the quantity of crystal within a given cell could be an important variable, too. At this time, as discussed in the paper, the sequence of events whereby an internalized crystal could be incorporated into a renal stone remains speculative.

C.F. Verkoelen: The renal nephron is composed of many different segments each of which is composed of different cell types that perform specific cellular functions. In most of the experiments described in this paper, BSC-1 cells were used. What is known about the origin of this cell line and do the authors believe that this cell line is representative for all these different cell types with respect to their response to crystals?

Authors: BSC-1 cells are a non-transformed kidney epithelial cell line derived from the African green monkey. Although the nephron segment from which BSC-1 cells originated is unknown, available evidence suggests that their phenotype is closely related to cells of the distal nephron. For example, these cells respond to vasopressin as a mitogen at concentrations 200-fold less than do fibroblasts {Walsh-Reitz M, Toback FG (1983) Vasopressin stimulates growth of renal epithelial cells in culture. Am J Physiol **245**: C365-C370}, contain IGF-I mRNA which is expressed by cells of the collecting duct (as determined by PCR (polymerase chain reaction) in a collaborative experiment with Dr. Donald Steiner, Depts. Biochem. and Mol. Biol., and Medicine, Univ. of Chicago, unpublished results), but lack erythropoietin mRNA, which is thought to be produced by proximal tubule or interstitial cells (as determined by PCR in a collaborative experiment with Dr. Eugene Goldwasser, Dept. Biochem. and Mol. Biol., Univ. of Chicago, unpublished results). Many of our experimental observations have been duplicated in canine renal epithelial cells of the MDCK line, also thought to be derived from the distal nephron [33, 35, 37]. Therefore, we believe that the response of BSC-1 cells to COM crystals is likely similar to that of cells of the distal nephron *in vivo*, which has been described in our previous publications.

C.R. Scheid: Your method for scoring crystal uptake by cells requires trypsinization, which may affect binding; are similar results found with subconfluent cultures?

Authors: Similar experiments have not been attempted in subconfluent cultures.

C.R. Scheid: Your data suggest that COM crystals bind preferentially to renal epithelial cells and that other crystal types bound less well, although comparable

amounts of crystals, i.e., similar in terms of μg/ml, were added. Were all crystals in the same size range? This could be a factor in successful endocytosis and also present steric problems for binding. Did you determine whether the binding was specific, i.e., could you prevent binding by the addition of unlabeled crystals; alternately, could COM crystals be displaced by HA?
Authors: All crystals were of a similar size (1-10 μm), as documented in our cited publications. We have not attempted to duplicate the experiments of Riese *et al.* [46] in which HA and COM crystals competitively inhibited the adhesion of one another.

C.R. Scheid: Your finding that cytokeratin 8 reorganization occurs in all cells after COM treatment, whether cells took up crystals or not, is quite interesting. Is there any further information as to the nature of the signaling molecule(s) involved here?
Authors: The finding that cytokeratin 8 reorganization occurred in all cells of a monolayer of BSC-1 cells after COM crystal addition suggests that a secondary signalling mechanism is triggered, but we do not have additional information regarding the identity of the signal(s).

C.R. Scheid: There is an apparent dissociation between the mitogenic effects of crystals and the effects on gene expression: HA was nearly as effective as COM at increasing cell numbers but had no effect on PAI-1 or PDGF. Do you have data that would explain this?
Authors: It is intriguing that although COM and HA crystals are both perceived as mitogens by BSC-1 cells, only COM crystals stimulated increased expression of the genes we studied that could contribute to interstitial fibrosis (e.g., PAI-1). We do not have additional information on specific underlying signalling events, but the results do suggest that crystals rely on different mechanisms to stimulate proliferative and non-proliferative responses by the cells.

S.R. Khan: Is it possible that crystal adherence and endocytosis are protective responses by cells of the renal epithelium? Perhaps these are the means for the kidneys to neutralize crystals by either the lysosomal activity inside the cells or pushing it into the interstitium where inflammatory processes take over.
Authors: We agree with Prof. Khan that the role of crystal endocytosis in the pathogenesis of renal stone formation remains to be determined. The ultimate fate of internalized CaOx crystals is a key issue, and one could hypothesize that, in some circumstances, crystal uptake could serve as a defense mechanism to eliminate crystals from the kidney. Additional study is required to explore these possibilities.

M.D.I. Gohel: Glycosaminoglycans are diverse in terms of size and charge; please comment on the relationship of charge to the inhibition of COM crystals in Figure 3?
Authors: No direct measurements of the net charge of the glycosaminoglycans depicted in Figure 3 were made. Although we agree with the reviewer that the anionic character of these molecules appears to be crucial for an anti-adherence effect, it is notable that diverse heparin and chondroitin sulfate disaccharides had no effect on COM crystal binding to BSC-1 cells [37]. Therefore, in addition to charge, the structure of these molecules is also an important determinant of their function.

M.D.I. Gohel: THP has a dual role of acting as a weak inhibitor of crystal aggregation at concentrations of $< 10^{-6}$ M and ionic strengths ≤ 0.16 and a promoter at higher strengths and concentrations. During the COM attachment studies, did the authors notice this differential role played by the THP?
Authors: THP was added directly to tissue culture medium that had the following composition: pH 7.4, Na^+ 155 mM, Ca^{2+} 1.8 mM. Under these conditions, the glycoprotein would not be expected to self-aggregate, as described by Hess [18].

M.D.I. Gohel: Could the mitogenic activity of the internalized COM crystals be due too the toxicity effects in the medium?
Authors: In control experiments, media exposed to COM crystals but no cells for 3 days at 37°C in a culture dish had no effect on proliferation of BSC-1 cells when this conditioned medium was subsequently placed on a cell monolayer [35]. Therefore, it appears unlikely that dissolution of COM crystals and release of calcium and/or oxalate into the medium mediates the proliferative effect.

M.D.I. Gohel: Transient CaOx crystals in the renal tubules are likely to be of the dihydrate-type (COD), as frequently seen in urine samples. Have the authors looked into the attachment of COD crystals, which may have different attachment characteristics than COM and is more likely to be the crystal in the *in vivo* situation?
Authors: It is noteworthy that the molecular array of the (101) face of COM crystals is closely related to that of the {100} face of COD {Deganello S (1993) The interaction between nephrocalcin and Tamm-Horsfall proteins with calcium oxalate dihydrate. Scanning Microsc **7**: 1111-1118}. Therefore, similar atomic arrays could mediate adhesion of both COM and COD crystals to the tubular cell surface. However, quantitative studies of adhesion of COD crystals to BSC-1 cells have not been performed.