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Renal epithelial cells rapidly bind and internalize calcium oxalate monohydrate crystals

(nephrolithiasis/endocytosis/cytoskeleton/actin)

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ABSTRACT **Renal tubular fluid is supersaturated with calcium and oxalate ions, which can nucleate to form crystals of calcium oxalate monohydrate (COM), the most abundant constituent of kidney stones. However, the mechanisms by which nascent crystals are retained in the nephron and then grow into kidney stones are unclear. An interaction of COM crystals with the surface of renal epithelial cells could be a critical initiating event in nephrolithiasis. To investigate this possibility we used cultures of monkey kidney epithelial cells (BSC-1 line) as a model system and found that [^{14}C]COM crystals bound to the cell surface within seconds. Scanning electron microscopy revealed that crystals bind first to apical microvilli, which subsequently migrate over the crystalline surface. When visualized by transmission electron microscopy, intracellular crystals were located within vesicles. Cytoskeletal responses to crystal uptake were sought by immunofluorescence microscopy, which revealed concentration of F-actin at sites of crystal contact as well as a generalized reorganization of the intermediate filament network containing cytokeratin 8. Uptake of COM crystals did not adversely affect renal epithelial cell growth, and internalized crystals were apparently distributed to daughter cells during division. Rapid adherence of COM crystals to the apical surface of tubular epithelial cells could promote crystal retention in the kidney. Elucidation of factors that regulate this process may provide insight into the pathogenesis of nephrolithiasis.**

Although nephrolithiasis is a common disease, the mechanisms by which stones develop in the kidney are poorly understood. Renal tubular fluid is supersaturated with calcium and oxalate ions, which can nucleate to form crystals of calcium oxalate monohydrate (COM). Once formed, it is not clear how these crystals are retained in the nephron and produce calculi. Calculations based on the rate of crystal growth and flow of tubular fluid suggest that a nascent crystal would not become large enough to occlude a tubule lumen during the time required for transit through the nephron (1). Either several small crystals could aggregate to form a mass large enough to block a tubule or small crystals could bind to the tubular epithelial cell surface; otherwise, crystals would leave the nephron suspended in the flowing tubular fluid and kidney stones would not form.

We have observed renal deposits of calcium oxalate crystals in a patient with primary hyperoxaluria after kidney–liver transplantation when the high body oxalate load was being excreted by the transplanted kidney (2). Crystals were observed within tubular epithelial cells and were associated with proliferation and formation of multinucleated giant cells. These observations in human kidney tissue suggest that renal epithelial cells can bind and internalize calcium oxalate crystals. To study this cell–crystal interaction *in vitro*, we have used high-density, quiescent cultures of nontransformed monkey renal epithelial cells (BSC-1 line) to simulate the tubular epithelium (3, 4). Crystals of COM are internalized by the cells and stimulate their proliferation, thereby duplicating our observations in the transplanted human kidney and validating this model system for the study of cell–crystal interactions in the nephron *in vivo*. Crystal and cell-type specificity are observed, and diverse agents such as urinary Tamm–Horsfall glycoprotein regulate the cell–crystal interaction. The aim of the present study was to identify early structural and functional changes at the kidney epithelial cell surface during an interaction with a COM crystal and thereby gain fresh insight into the pathogenesis of nephrolithiasis.

MATERIALS AND METHODS

Subconfluent or high-density, quiescent cultures of renal epithelial cells of the nontransformed African green monkey line (BSC-1) or Madin–Darby canine kidney (MDCK) cells were used (3). Crystals of COM (1–2 μm), both unlabeled and radiolabeled with [^{14}C]oxalic acid to a specific activity of 24,000 cpm/mg, were prepared by Y. Nakagawa (University of Chicago) as described (3). Before use, crystals were sterilized by heating to 180°C overnight and then suspended in distilled water to form a slurry from which they were added to the culture medium (3). X-ray crystallography, performed by S. Deganello (University of Chicago), demonstrated that heating did not alter the structure of COM crystals.

[^{14}C]COM crystals (10–300 μg/ml; 2.4–70.8 μg/cm^2 cell surface) were added to high-density, quiescent cultures in 60-mm dishes (Nunc) to define the kinetics of association between a cell and crystal. After a specified period, the medium was aspirated, and the monolayer was washed three times with phosphate-buffered saline (PBS; 5 ml). Each culture was inspected under a microscope and the number of cells with adherent crystals was counted in five separate fields. Subsequently, the cell monolayer with adherent crystals was scraped directly into a scintillation vial containing 6 M HCl (0.5 ml), 4.5 ml of Ecoscint (National Diagnostics) was added, and the amount of radioactivity was measured. To investigate the effect of crystal exposure for up to 15 days on renal epithelial cells, COM crystals (50 μg/ml) were added to near-confluent cultures of BSC-1 cells (10^6 cells per 60-mm dish) containing 0.5% calf serum. Every 4 days thereafter, the medium was aspirated and replaced with fresh medium containing 0.5% calf serum with no additional crystals. At 1, 8, and 15 days after addition of crystals, the medium was aspirated, and a solution of crystalline trypsin was used to detach the cells, which were then inspected under a microscope as described (4). The total number of cells in each culture was counted with a hemocytometer, and 100 cells from each culture were scored for the presence of internalized crystals.

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Abbreviations: COM, calcium oxalate monohydrate; TEM, transmission electron microscopy; SEM, scanning electron microscopy.

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To study the cell–crystal interaction by transmission electron microscopy (TEM) or scanning electron microscopy (SEM), high-density cultures of BSC-1 cells were grown either on plastic 60-mm dishes (Permanox; Nunc) or on glass coverslips, respectively. Before and at specified times after addition of COM crystals (200 μg/ml), the medium was aspirated and cells were fixed with Karnofsky solution and postfixed with osmium tetroxide as described (3). To optimally preserve intracellular architecture for TEM, pellets were prepared by scraping cells into an Eppendorf tube and collecting them by centrifugation at 3000 × *g* before fixation. For TEM, fixed cells were dehydrated in increasing concentrations of ethanol (35% to absolute) and embedded in Epon epoxy resin. Ultrathin sections were cut on a Sorvall MT2-B ultramicrotome, stained for 1 hr with uranyl acetate and for 3 min with lead citrate, and then examined at 80 kV with a Siemens 101 electron microscope. For SEM, cells fixed on glass coverslips were dehydrated in increasing concentrations of ethanol (10–100%). Each specimen was air dried, mounted on a stub, coated with gold for 4 min, and examined with an ETEC scanning electron microscope at 40 kV.

Cytoskeletal structures were examined by fluorescence microscopy in high-density, quiescent cultures before and after exposure to COM crystals. At specified times after addition of COM crystals (100 μg/ml), the monolayer was rinsed with PBS and the cells were fixed with freshly prepared 0.037 M sodium phosphate buffer containing 0.01 M sodium periodate, 0.075 M lysine, and 2% paraformaldehyde for 15 min at room temperature (5). To stain F-actin, the coverslips were incubated with fluorescein isothiocyanate/phalloidin (Sigma) for 20 min at room temperature in a humidified chamber, rinsed with PBS, and mounted. To visualize cytokeratin 8, cells fixed on coverslips were incubated for 1 hr with a specific anti-cytokeratin 8 monoclonal mouse IgG (gift from Werner Franke, German Cancer Research Center, Heidelberg), fluorescein-conjugated secondary antibody was added for 1 hr, and the coverslips were rinsed with PBS and mounted (5). After viewing under conventional immunofluorescence microscopy, fluorescence intensities on the coverslips stained for cytokeratin 8 were determined by acquisition of digitized video images, which were subsequently analyzed with Image 1 software (Universal Imaging, Media, PA).

RESULTS

The capacity of renal epithelial cells to bind urinary crystals was studied in high-density, quiescent cultures of BSC-1 cells. Initial adhesion of [^{14}C]COM crystals was maximal 15 sec after 100 μg of crystal per ml of medium was added and within 30 sec when 300 μg/ml was used (Fig. 1*A*). Inspection by light microscopy showed that the presence of visible crystals correlated with cell-associated radioactivity (Fig. 1*B*). When measured 1 min after addition, the quantity of adherent crystals increased linearly as a function of added crystal over the concentration range used (12.5–300 μg/ml). Binding of [^{14}C]COM crystals to high-density, quiescent cultures of MDCK cells was observed minutes after addition to the culture medium, with a linear increase in crystal binding when up to 300 μg/ml was added (data not shown). Thus, COM crystals can adhere within seconds to the surface of kidney epithelial cells in a concentration-dependent manner.

SEM was used to examine the structural correlates of COM crystal binding to the apical membrane of BSC-1 cells. Under low power, the outlines of individual cells, nuclei, and surface microvilli were seen, as well as small adherent crystal aggregates (Fig. 2*A*, arrows). Higher magnification revealed contact between microvilli and the crystal surface (Fig. 2*B*, arrow). At the base of the same microvillus, small cellular extensions could be seen over the surface of the crystal (Fig. 2*C*). In other instances, extended microvilli covered a substantial portion of the crystal (Fig. 2*D*). These microvillar processes appeared to subsequently coalesce and completely cover the crystal (Fig. 2*E*). At later times, apparent crystal aggregates were observed immediately beneath the plasma membrane (Fig. 2*F*). Microvilli on the surface of macrophages appear to contribute to phagocytosis in a similar manner (6).

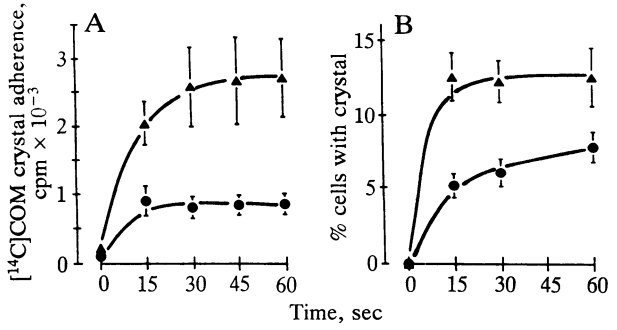

FIG. 1. Time and concentration dependence of COM crystal binding to renal epithelial cells in culture. (*A*) Maximal crystal binding, as evidenced by cell-associated ^{14}C, was detected after 15 sec (100 μg/ml; circles) or 30 sec (300 μg/ml; triangles). (*B*) Inspection by light microscopy demonstrated that the number of cells binding a crystal was correlated with cell-associated radioactivity.

TEM was used to visualize intracellular changes as COM crystals were engulfed. Crystals adherent to microvilli were noted (Fig. 3 *A* and *B*). Microvillar processes appeared to extend sequentially to occupy a sizable portion of the crystal surface (Fig. 3*C*). Inside cells, crystals appeared within membrane-lined vacuoles (Fig. 3 *D* and *E*). Lysosomes were located in the vicinity of intracellular crystals at 3 hr (data not shown), and after 12 hr small crystals were seen within the organelle (Fig. 3*F*, arrows).

Actin filaments are necessary for phagocytosis by other cell types such as macrophages, and particulate uptake can be blocked by cytochalasin B (7). To determine whether actin filaments were associated with the internalization of COM crystals, phalloidin was used, which selectively binds to F (filamentous)-actin but not to G (globular)-actin. At 1 hr after exposure to COM crystals, increased phalloidin staining was apparent in a region of the cell just beneath the crystal; these changes were maximal at 3 hr (Fig. 4). Phalloidin staining appeared to follow the outline of the crystal during engulfment, suggesting a role for actin during internalization.

We also sought changes in other cytoskeletal filament systems. Staining of cells exposed to COM crystals with a monoclonal antibody to tubulin did not reveal any morphologic alterations (data not shown). Previous studies revealed that some compounds that are mitogenic for BSC-1 cells caused surprisingly rapid alterations in the cytokeratin components of the intermediate filament network (5). We therefore studied the effect of COM crystals on cytoskeletal intermediate filaments by using a monoclonal antibody to cytokeratin 8. In control cells, this protein stained most intensely in a perinuclear zone (Fig. 5*A*, phase-contrast microscopy; Fig. 5*B*, immunofluorescence microscopy) but redistributed to a diffuse cytoplasmic fiber pattern 8 hr after exposure to COM crystals (Fig. 5*C*, phase-contrast microscopy; Fig. 5*D*, immunofluorescence microscopy). Cytokeratin 8 relative fluorescence intensity was measured on 16 fields of 200 cells each as described. Mean relative fluorescence intensity was 14% above control at 1 hr ($P < 0.01$), 93% after 3 hr ($P < 0.001$), and maximally increased 336% above control 8 hr after addition of COM crystals ($P < 0.001$) (data not shown). Cytokeratin 8 reorganization was observed in all cells after addition of COM crystals and was not confined to those that internalized a crystal (Fig. 5*D*).

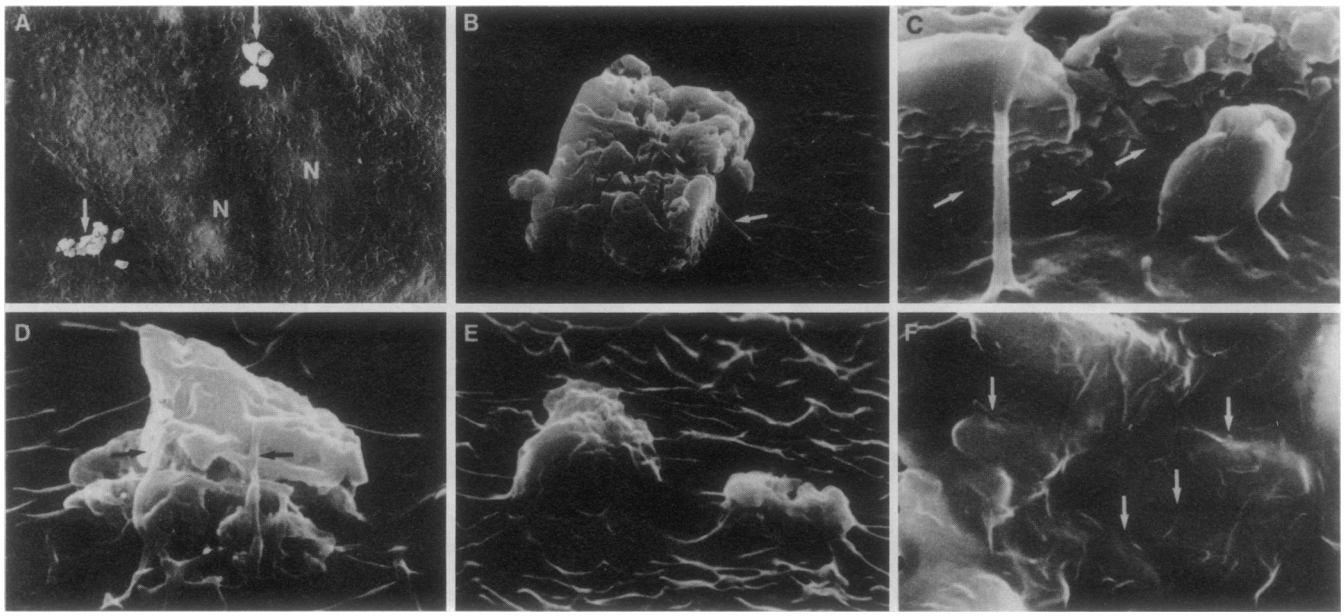

FIG. 2. Appearance of COM crystals on the surface of BSC-1 cells. (*A*) Low-power view demonstrates portions of several cells, nuclei (N), microvilli, and a few small COM crystal aggregates (arrows; 1-hr exposure). (×1200.) (*B*) Higher-power view of an individual crystal on the cell surface demonstrates a microvillar projection (arrow) extending over the surface of the crystal (30-min exposure). (×5250.) (*C*) Closer view of the base of the same microvillus as in *B* reveals several additional microvilli (arrows; 30-min exposure). (×15,850.) (*D*) Individual COM crystal with many large microvilli (arrows), which appear to cover the crystalline surface (30-min exposure). (×15,850.) (*E*) Two crystals appear to be completely surrounded by cellular projections (6-hr exposure). (×11,500.) (*F*) Aggregate of COM crystals under plasma membrane surface (arrows) after engulfment (6-hr exposure). (×7550.)

As COM crystals are mitogenic for BSC-1 cells (4), we wished to know the fate of crystals in cells going through mitosis. Subconfluent cultures of BSC-1 cells were prepared and crystals (50 μg/ml) were added on day 0. On day 1, the medium was changed to remove any nonadherent crystals. Seven or 14 days later, the total number of cells did not differ from control cultures not exposed to crystals (Fig. 6). Thus, the presence of intracellular crystals did not adversely affect

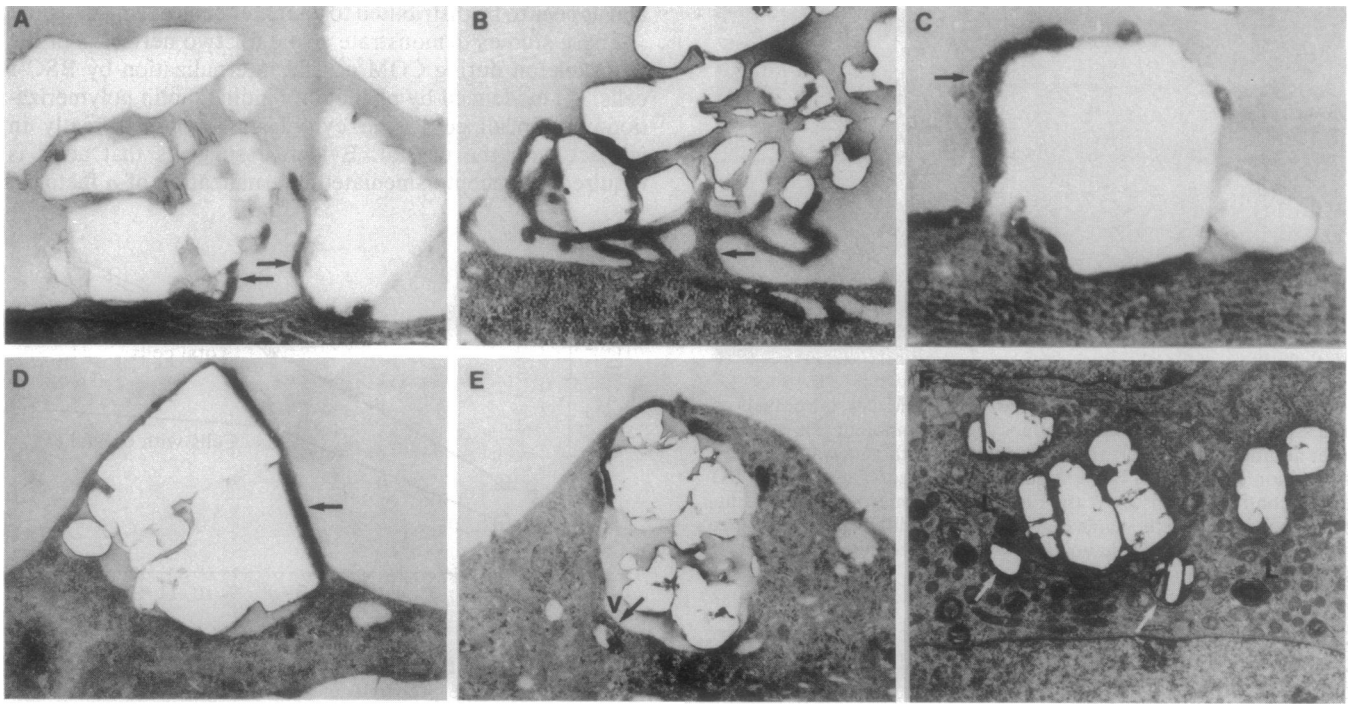

FIG. 3. TEM of COM crystal binding to the apical BSC-1 cell surface and subsequent uptake. (*A*) Microvilli can be seen contacting the surface of a crystal aggregate in multiple locations (arrows) (30-min exposure). (×3900.) (*B*) Large cellular projection (arrow) extends from the surface of the cell over a COM crystal aggregate (15-min exposure). (×5700.) (*C*) Smaller crystal appears partially engulfed by a cellular projection (arrow) (15-min exposure). (×10,300.) (*D*) Crystal can be seen lying just below the plasma membrane, covered by a thin rim of cytoplasm (arrow) and protruding from the cell surface (15-min exposure). (×3100.) (*E*) Crystal aggregate lies within a membrane-lined vesicle (V) inside a cell (15-min exposure). (×2200.) (*F*) Cell contains several crystals and multiple lysosomal bodies (L) are also present, two of which appear to contain small crystals (arrows) (12-hr exposure). (×1700.)

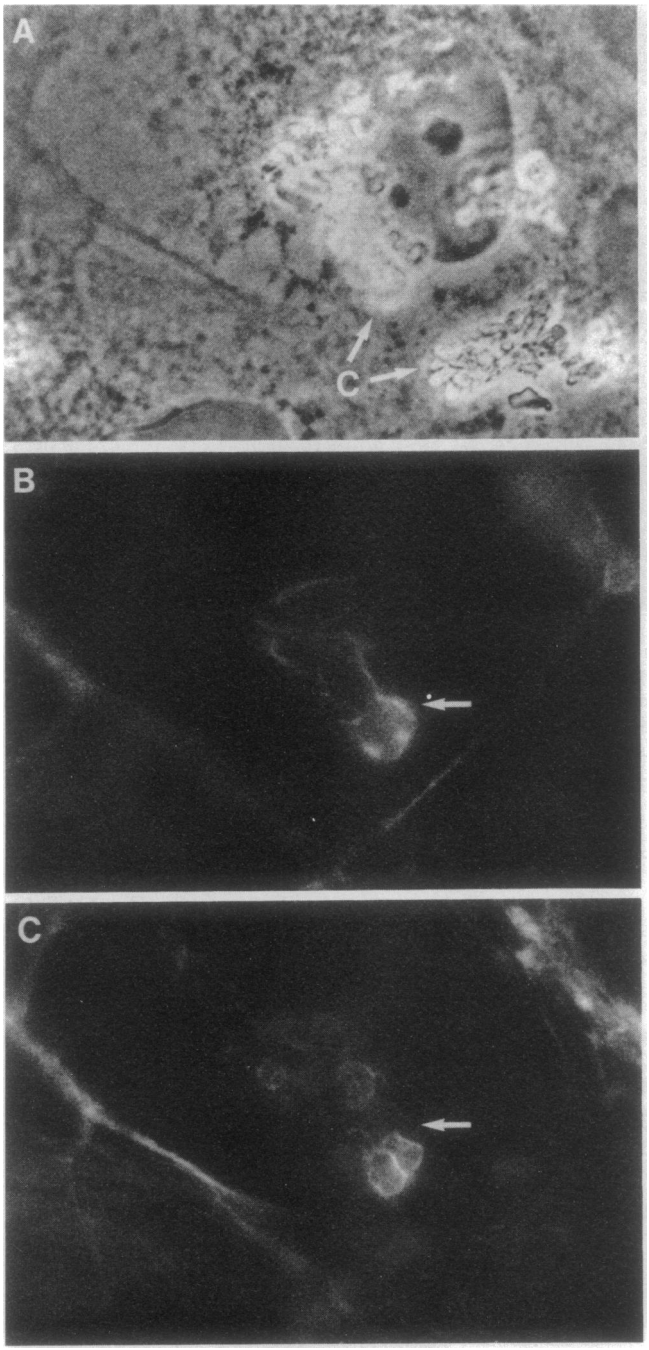

FIG. 4. Actin changes associated with COM crystal uptake as assessed by using fluorescein isothiocyanate phalloidin. Three hours after addition of COM crystals (C), phalloidin staining was localized in a region of a cell just below a small crystal aggregate (arrow). (*A*) Phase-contrast microscopy. (*B*) Fluorescence microscopy. (*C*) Fluorescence image of the same cell at a slightly different focal plane; the manner in which phalloidin staining surrounded the crystal surface is shown. (×3650.)

cell growth. Furthermore, the number of cells in the culture containing one or more crystals increased between 1 and 7 days ($P < 0.001$), although no additional crystals were added after day 0 (Fig. 6), demonstrating that internalized crystals were passed on to daughter cells during division.

DISCUSSION

These results indicate that crystals of COM bind rapidly in a concentration-dependent manner to the apical surface of cells of two renal epithelial lines. Microvilli may provide the initial

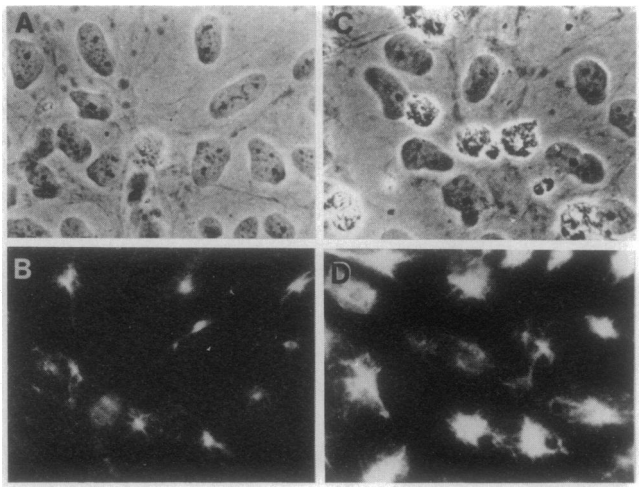

FIG. 5. Intermediate filament (cytokeratin 8) reorganization in BSC-1 cells after exposure to COM crystals. Control cells exhibit intense cytokeratin 8 staining only in a star-shaped pattern originating from a perinuclear zone extending toward the cell periphery. (*A*) Phase-contrast microscopy. (*B*) Immunofluorescence microscopy. After 8 hr of exposure to COM crystals, overall cytokeratin 8 staining intensity is increased and the network appears extended throughout the cytoplasm. (*C*) Phase-contrast microscopy. (*D*) Immunofluorescence microscopy. (×340.)

binding site and appear to participate in a process that results in the eventual internalization of the crystal into a membrane-lined vesicle. Cytoskeletal participation in this process includes the concentration of F-actin beneath the plasma membrane at the site of the crystal–cell interaction, reorganization of the intermediate filament cytokeratin 8 in all cells of the monolayer, and no apparent role for microtubules. Internalized crystals do not adversely affect cell proliferation and appear to be distributed to daughter cells during division.

These studies demonstrate a role for two networks of the cytoskeleton during COM crystal internalization by BSC-1 cells. As evidenced by phalloidin binding, actin polymerization was localized in the cytoplasmic region directly in contact with the crystal. Evidence suggests that actin is required for receptor-mediated internalization of α factor in

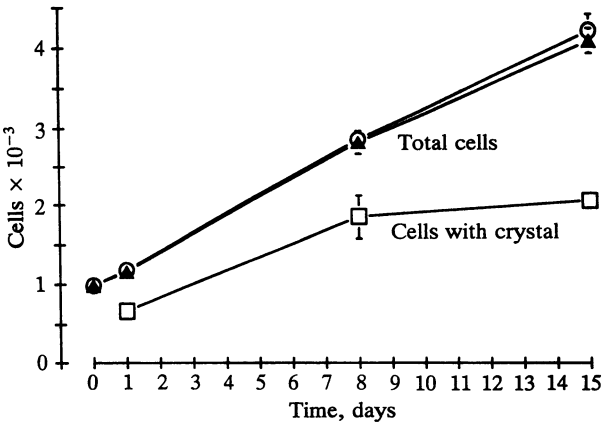

FIG. 6. Fate of intracellular COM crystals. Subconfluent cultures of BSC-1 cells were prepared and exposed to COM crystals (50 μg/ml) on day 0. Nonadherent crystals were removed by a medium change on day 1. One and 2 weeks later, the number of cells and the presence of intracellular crystals were assessed. Total number of cells in cultures exposed to 50 μg of COM crystals per ml (solid triangles) did not differ from control (open circles) 7 or 14 days after exposure. Total number of cells containing intracellular crystal increased from 0.7×10^6 on day 1 to 1.8×10^6 on day 7 (open squares) ($P < 0.001$).

yeast (8) and for the scission of vesicles from the apical membrane of MDCK cells in culture (9). Since actin filaments are contained in the core of microvilli (10) and COM crystals appeared to bind specifically to apical surface projections, these two morphologic features of crystal engulfment may be linked. The potential importance of microvilli during COM crystal binding and uptake has not been previously reported. Changes observed in the intermediate filament cytokeratin 8 of BSC-1 cells after crystal uptake seem to be a generalized response of the cell to crystal internalization and not responsible for mediating the process. After 8 hr of exposure to COM crystals, all of the cells in the monolayer displayed alterations in the cytokeratin 8 network, not just those that contained a crystal, suggesting that cell-to-cell communication had occurred in response to crystal uptake, perhaps via the release of an autocrine factor. Similar changes in cytokeratin 8 staining were seen in BSC-1 cells after exposure to the renal cell mitogen ADP (5) so that intermediate filament alterations after COM crystal uptake may be related to the mitogenic response that ensues (4).

BSC-1 cells appear to survive and divide in culture despite the presence of internalized COM crystals, providing evidence that the crystals are not toxic for these renal cells. In fact, COM crystals are mitogenic for cultured renal epithelial cells, a unique property not shared by another calcium-containing crystal (brushite) or latex beads (1). The uptake of COM crystals by BSC-1 cells is a regulated event that can be modified by diverse signals (3). The mitogens epidermal growth factor, ADP, and calf serum each increase COM crystal endocytosis, whereas urinary Tamm–Horsfall glycoprotein, heparin, transforming growth factor $\beta 2$, and the tetrapeptide Arg-Gly-Asp-Ser (RGDS) inhibit it (3). Thus, renal epithelial cells respond in a specific pattern to a crystal commonly found in urine, and these responses can be modified by extracellular signals.

The mechanisms by which urinary crystals are retained in the kidney and grow into a kidney stone are not known. Calculations based on the concentrations of ions in tubular fluid and the maximal rate of calcium oxalate crystal growth suggest that a crystal could not reach a size large enough to occlude a tubular lumen in the time necessary for transit from the proximal tubule to the end of the collecting duct (1). Thus, factors that allow small crystals to become anchored to the cell surface and remain in contact with supersaturated tubular fluid could be important determinants of stone growth. The micrographs in this manuscript, together with our previous investigations (3, 4), suggest that COM crystals that form in tubular fluid may preferentially adhere to microvilli on the apical surface of tubular epithelial cells. Previous studies in BSC-1 cells (3) and in primary cultures of rat inner medullary collecting duct cells (11) suggest that COM crystals bind to specific receptors on the cell surface, the number and character of which may be influenced by the distribution of apical and basolateral proteins on the plasma membrane (12). Receptors that bind COM crystals on tubular cells may be only minimally exposed under normal circumstances but could increase in number under conditions of injury, as has been demonstrated for denuded rat bladder epithelium (13). Crystal-binding receptors could ordinarily mediate cellular adhesion to the extracellular matrix (3) or to a nonbiologic substrate such as a crystal (14) or could contribute to migration of epithelial cells (15). RGDS blocked adhesion of A6 kidney cells to specific crystalline surfaces and also impeded uptake of COM crystals by BSC-1 cells (3, 14). Agents that retarded (RGDS, heparin, transforming growth factor $\beta 2$) or enhanced (ADP, epidermal growth factor) migration of BSC-1 cells in culture also diminished or increased COM crystal uptake, respectively (3, 15). Therefore, the processes of epithelial cell adhesion to and migration on the basement membrane may be linked to COM crystal binding and uptake, perhaps by using similar receptors. If renal epithelial cells *in vivo* behave similarly to BSC-1 cells in culture, anchoring of newly formed COM crystals to apical microvilli could promote crystal retention in the kidney either through continued crystal growth or by aggregation of additional crystals. The results suggest that cell surface structures such as microvilli can contribute to crystal retention and nephrolithiasis in primary hyperoxaluria before and after renal transplantation and possibly in other stone-forming states.

In summary, COM crystals rapidly bind to the surface of renal epithelial cells and are internalized. This process, by promoting crystal retention within the kidney, could be a key event in nephrolithiasis.

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1. Finlayson, B. & Reid, S. (1978) *Invest. Urol.* **15**, 442–448.
2. Lieske, J. C., Spargo, B. & Toback, F. G. (1992) *J. Urol.* **148**, 1517–1519.
3. Lieske, J. C. & Toback, F. G. (1993) *Am. J. Physiol.* **264**, F800–F807.
4. Lieske, J. C., Walsh-Reitz, M. M. & Toback, F. G. (1992) *Am. J. Physiol.* **262**, F622–630.
5. Kartha, S., Atkin, B., Martin, T. E. & Toback, F. G. (1992) *Exp. Cell Res.* **200**, 219–226.
6. Speert, D. P. & Gordon, S. (1992) *J. Clin. Invest.* **90**, 1085–1092.
7. Axline, S. G. & Reaven, E. P. (1974) *J. Cell Biol.* **62**, 647–659.
8. Kubler, E. & Riezman, H. (1993) *EMBO J.* **12**, 2855–2862.
9. Gottlieb, T. A., Ivanov, I. E., Adesnik, M. & Sabatini, D. D. (1993) *J. Cell Biol.* **120**, 695–712.
10. Molitaris, B. A. & Nelson, W. J. (1990) *J. Clin. Invest.* **85**, 3–9.
11. Reise, R. J., Reise, J. W., Kleinman, J. G., Wiessner, J. H., Mandel, G. S. & Mandel, N. S. (1988) *Am. J. Physiol.* **255**, F1025–F1032.
12. Reise, R. J., Mandel, N. S., Wiessner, J. H., Mandel, G. S., Becker, C. G. & Kleinman, J. G. (1992) *Am. J. Physiol.* **262**, F177–F184.
13. Khan, S. R., Cockrell, C. A., Finlayson, B. & Hackett, R. L. (1984) *J. Urol.* **138**, 557–562.
14. Hanein, D., Sabanay, H., Addadi, L. & Geiger, B. (1993) *J. Cell Sci.* **104**, 275–288.
15. Kartha, S. & Toback, F. G. (1992) *J. Clin. Invest.* **90**, 288–292.