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Direct nucleation of calcium oxalate dihydrate crystals onto the surface of living renal epithelial cells in culture

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Background. The interaction of the most common crystal in human urine, calcium oxalate dihydrate (COD), with the surface of monkey renal epithelial cells (BSC-1 line) was studied to identify initiating events in kidney stone formation.

Methods. To determine if COD crystals could nucleate directly onto the apical cell surface, a novel technique utilizing vapor diffusion of oxalic acid was employed. Cells were grown to confluence in the inner four wells of 24-well plates. At the start of each experiment, diethyloxalate in water was placed into eight adjacent wells, and the plates were sealed tightly with tape so that oxalic acid vapor diffused into a calcium-containing buffer overlying the cells.

Results. Small crystals were visualized on the cell surface after two hours, and by six hours the unambiguous habitus of COD was confirmed. Nucleation onto cells occurred almost exclusively via the (001) face, one that is only rarely observed when COD crystals nucleate onto inanimate surfaces. Similar results were obtained when canine renal epithelial cells (MDCK line) were used as a substrate for nucleation. Initially, COD crystals were internalized almost as quickly as they formed on the apical cell surface.

Conclusions. Face-specific COD crystal nucleation onto the apical surface of living renal epithelial cells followed by internalization is a heretofore unrecognized physiological event, suggesting a new mechanism to explain crystal retention within the nephron, and perhaps kidney stone formation when this process is dysregulated or overwhelmed.

The events that initiate renal stone development are not well understood. Urine is commonly supersaturated with calcium and oxalate ions that favor the formation of calcium oxalate crystals, the most common constituent of kidney stones [1]. However, nucleation of ions from renal tubular fluid followed by growth of a calcium oxalate crystal cannot proceed quickly enough to produce a particle of sufficient size to occlude a tubule lumen, given the time required for these processes and the rate of fluid flow [2]. It has been postulated that after nucleating within the tubule lumen of a stone-forming individual, a nascent crystal either aggregates with other crystals to form a mass large enough to occlude the nephron or adheres to the tubular epithelium. To investigate cell-crystal interactions, we and others have characterized the binding of preformed calcium oxalate crystals to the surface of renal epithelial cells and cellular responses that follow [3–7], which may be critical initiating events in nephrolithiasis.

Although information is increasingly available about extracellular matrix proteins that interact with the basolateral cell surface [8], little is known about adhesive molecules on the apical plasma membrane. At the molecular level, adhesion of a cell to a substratum requires the interaction of cytoplasmic, transmembrane and extracellular proteins to form stable contact sites [9]. When an individual crystal forms within a nephron and makes contact with a tubular cell, adhesive molecules distributed on its luminal surface could be critical determinants of whether the crystal binds and is retained in the kidney. We have previously demonstrated selective bonding between the apical surface of renal epithelial cells in culture and exogenous calcium oxalate dihydrate (COD) crystals [10], the most common crystal in human urine [11], via the (100) face and its symmetry-related counterparts. As preformed calcium oxalate monohydrate (COM) crystals also avidly adhere to renal cells in culture [5–7] and are structurally similar to COD [12], a set of molecules on the apical plasma membrane appears to interact with molecular arrays on the surfaces of these two crystals. Furthermore, COD crystals are metastable and once formed tend to undergo a phase transition to COM, which is the thermodynamically stable hydrate and most abundant crystal found in kidney stones [1]. In this way the association of COD crystals with renal cells could result in formation of COM stones. Nucleation of different crystals onto cell surfaces could be an important event during physiologic and pathological biomineralization processes in diverse tissues [13–17]. To gain fresh insight into events that initiate calcium oxalate renal calculi, we set out to deter-

Key words: calcium oxalate, cell membrane, crystallization, crystallography, x-ray of kidney calculi, models of stones, structural defects.

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mine if calcium oxalate crystals could nucleate onto the renal epithelial cell surface.

METHODS

Cell culture

To determine if COD crystals can nucleate onto the apical surface of living renal cells, we developed conditions to promote nucleation and growth of high-quality COD crystals by vapor diffusion of oxalic acid inside sealed multi-well culture dishes [10, 18]. In this model system crystal nucleation proceeds more slowly than might occur in supersaturated tubular fluid *in vivo*; however, large well-formed COD crystals (10 to 20 μm) that are readily amenable for structural and ultrastructural analysis are produced. Nontransformed African green monkey renal epithelial cells of the BSC-1 line [19] were plated and grown to confluence in the inner four wells of 24-well plates (6×4 well configuration, Falcon #3047; Becton Dickenson, Franklin Lake, NJ, USA) in Dulbecco-Vogt modified Eagle's medium containing 25 mM glucose (DMEM), 1% calf serum and 1.6 μM biotin at 38°C in a CO_2 incubator [3]. To start an experiment, medium bathing cells was replaced with a calcium-containing buffer (155 mM NaCl, 5.4 mM KCl, 100 mM glucose, 50 mM $CaCl_2$, 90 mM Tris, pH 7.4). Three to five well-formed COD crystals (10 to 20 μm in cross section) [10] were added as seeds to the periphery of each of these four wells with a pipette. Diethyloxalate (20 μl in 2 ml water) was put into each of the surrounding eight wells. Tris buffer (0.5 M, pH 7.4) was placed in four outer wells and the spaces between wells. The eight remaining wells, four at the far left and four at the far right edges of the plate were covered with electrical tape and were not used. Oxalic acid vapor, produced by hydrolysis of diethyloxalate, diffused into the calcium-containing buffer overlying cells because the plates were sealed with electrical tape for the duration of the experiment and maintained at 37°C. The pH in these wells remained at 7.4 due to Tris buffer in both the vapor and the liquid phase [18]. The maximal concentration of oxalate achieved by vapor diffusion into the buffer overlying the cells, measured at the conclusion of a representative 12-hour experiment using an oxalate diagnostic kit (Sigma), was 0.46 mM. This value is similar to that found in human urine [1].

Electron microscopy

For scanning electron microscopy (SEM), BSC-1 cells were grown on glass coverslips within the inner four wells of the 24-well Falcon plates. At specified times after initiation of COD crystal nucleation as described above, the buffer was aspirated and cells were fixed with Karnofsky solution [4]. Each specimen was air dried, mounted on a stub, coated with carbon by glow discharge, and examined both with an ETEC or JEOL (model JSM-5800LV) scanning electron microscope at 25 kV. X-ray microanalysis was carried out at 15 kV in energy dispersive mode (EDS) using the Oxford-Link ISIS-300 platform.

To study intracellular structures by transmission electron microscopy (TEM), at specified times after initiation of COD crystal nucleation the buffer was aspirated and cells were gently scraped from the wells into an Eppendorf tube that was subsequently centrifuged at 3000 *g*. The cell pellet was fixed with Karnofsky solution for one hour at 4°C, postfixed in osmium tetroxide, dehydrated in increasing concentrations of ethanol, and embedded in Epon epoxy resin as previously described [4]. Ultrathin sections were cut on a Sorvall MT2-B ultramicrotome, stained with uranyl acetate and lead citrate, and examined at 80 kV with a Siemens 101 electron microscope.

X-ray diffraction

Preliminary characterization and identification of nucleated crystals were carried out by polarized light and SEM as described previously [20]. Positive phase identification was achieved by X-ray diffraction using a Gandolfi camera (114.6 mM) and Ni-filtered CuKα radiation (40 kV, 20 mA). To unambiguously identify small crystals adherent to cells, electron diffraction patterns were taken at 100 kV using the same Siemens 101 electron microscope (camera length 770 mM). Diffraction patterns were indexed according to A.S.T.M. Fiche Number 17-541 [21]. Modeling of the atomic structure of COD was done using the atomic coordinates of Tazzoli and Domeneghetti [22] with the program ATOMS (ESM Software, Inc., Cincinnati, OH, USA).

If not indicated otherwise, compounds were purchased from Sigma Chemical Company (St. Louis, MO, USA).

RESULTS

Crystals rapidly nucleated and grew on the surface of BSC-1 cells within sealed multi-well dishes following diffusion of oxalate into the calcium-containing buffer overlying the cells. Under phase-contrast microscopy ($\times$200), a few crystals were seen on the apical cell surface after two hours (arrow, Fig. 1A), and by three hours more abundant nucleation was obvious (arrow, Fig. 1B). At six hours, the typical bypyramidal habitus of COD was unambiguous (Fig. 1C). Positive identification of a representative crystal as COD was achieved by x-ray diffraction using a Gandolfi camera (114.6 mM; 10^{-3} torr) and Ni-filtered CuKα radiation. In contrast, onset of detectable COD crystal nucleation onto the plastic surface of a culture dish in the absence of cells required at least six hours, and was random in orientation, in contrast to the uniform orientation of crystals nucleated onto the surface of cells (Fig. 1C). As nucleation is a random phenomenon, the observed rigorous control of crystal orientation, which included highly regulated morphology (Fig. 1C) and face specificity (see below), suggested that an interaction existed between the growing nuclei and atomic structures on the surface layers of the cells that functioned as a template. SEM at three hours (Fig. 1 D-F) revealed many crystals on the cell surface that appeared to be undergoing internalization (Fig. 1D), and extensions of cellular material were readily seen overlying

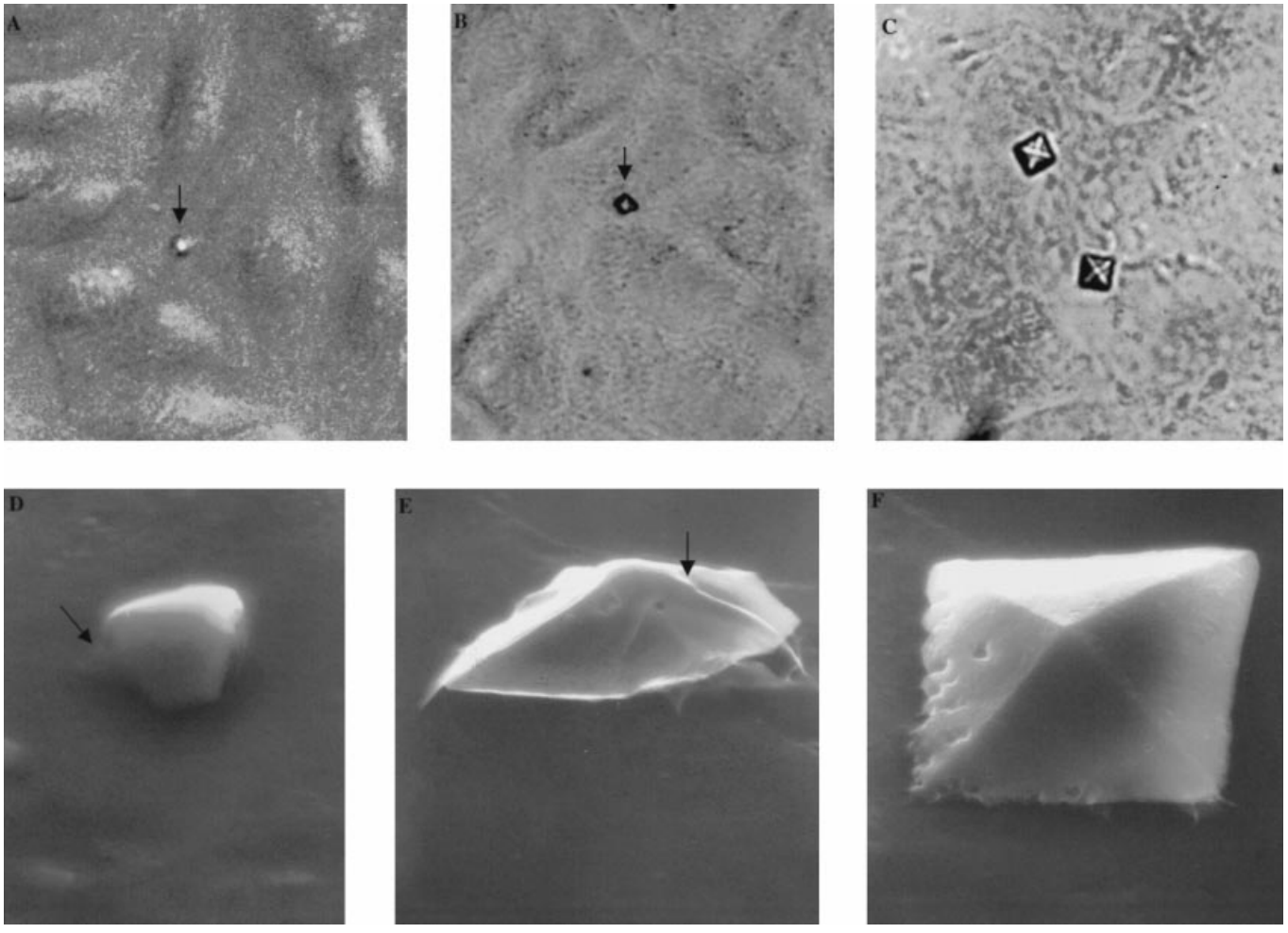

Fig. 1. Nucleation of calcium oxalate dihydrate (COD) crystals onto living BSC-1 cells. Nontransformed African green monkey renal epithelial cells (BSC-1 line) were grown to confluence in the inner four wells of a Falcon 24-well tissue culture dish in Dulbecco's-modified Eagle's medium containing 1% calf serum and 1.6 μM biotin. Medium bathing the cells was replaced with buffer (155 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 90 mM Tris, pH 7.4). In the surrounding 12 wells, diethyloxalate (20 μl in 2 ml water) was placed in the closest 8, and Tris buffer (0.5 M, pH 7.4) in the outer four wells. Tris buffer was then placed in the spaces between wells. The four wells at both the far right and left of the plate were covered with electrical tape and not used. Dishes were sealed with tape for the duration of the experiment and maintained at 37°C. Oxalic acid vapor produced by hydrolysis of diethyloxalate and buffered by Tris vapor, diffused into calcium-containing buffer overlying the cells allowing COD crystals to form. Small crystals were detected under phase-contrast microscopy by two hours (arrow, *A*, ×3200), and were larger after three hours (arrow, *B*, ×2000). At six hours, typical bypyramidal crystals of COD were easily identified (*C*, ×1600). Samples were also fixed in half-strength Karnofsky's solution at 4°C for 30 minutes, air-dried, and carbon coated for SEM. After 3 (*D*, ×8500) and 6 (*E*, ×3000) hours, nucleated crystals on the cell surface were partially (arrow, E) or completely covered (D) by the plasma membrane. While anchored to the cell surface, certain crystals not undergoing internalization permitted the well-formed habitus of COD to be seen at six hours (*F*, ×4500).

portions of the surface of certain larger crystals (Fig. 1E). The presence of smaller crystals inside cells was confirmed by TEM (see below). X-ray microanalysis of several of these crystals revealed that they contained calcium, oxygen and traces of carbon, but no phosphorous was detected. Therefore, the SEM, x-ray diffraction, and x-ray microanalysis data confirmed unequivocally that these crystals were COD. Under these experimental conditions, cells remained viable for at least 12 hours as confirmed by trypan blue exclusion. Measurements at six hour intervals confirmed that the pH of buffer overlying cells remained at 7.4. When, after a 12 hour experiment, buffer was replaced by DMEM containing 1% calf serum and plates were returned to an incubator at 37°C, cells continued to grow and divide. Thus, the cells on whose surfaces COD crystals nucleated did not appear damaged. When confluent MDCK cell monolayers (prepared as previously described [3]) were substituted for BSC-1 cells in this protocol, similarly oriented and equally well-developed COD crystals were visualized on their apical surfaces after five hours by phase-contrast microscopy (×400). Therefore, COD crystals can nucleate directly onto the surface of living renal cells and appear to be internalized almost as quickly as they form.

By twelve hours, numerous COD crystals were apparent on the surface of BSC-1 cells of the monolayer (Fig. 2A). The uniform habitus of these crystals indicated that nucleation and growth took place via a common face, in marked contrast to the random orientation of crystals nucleated on

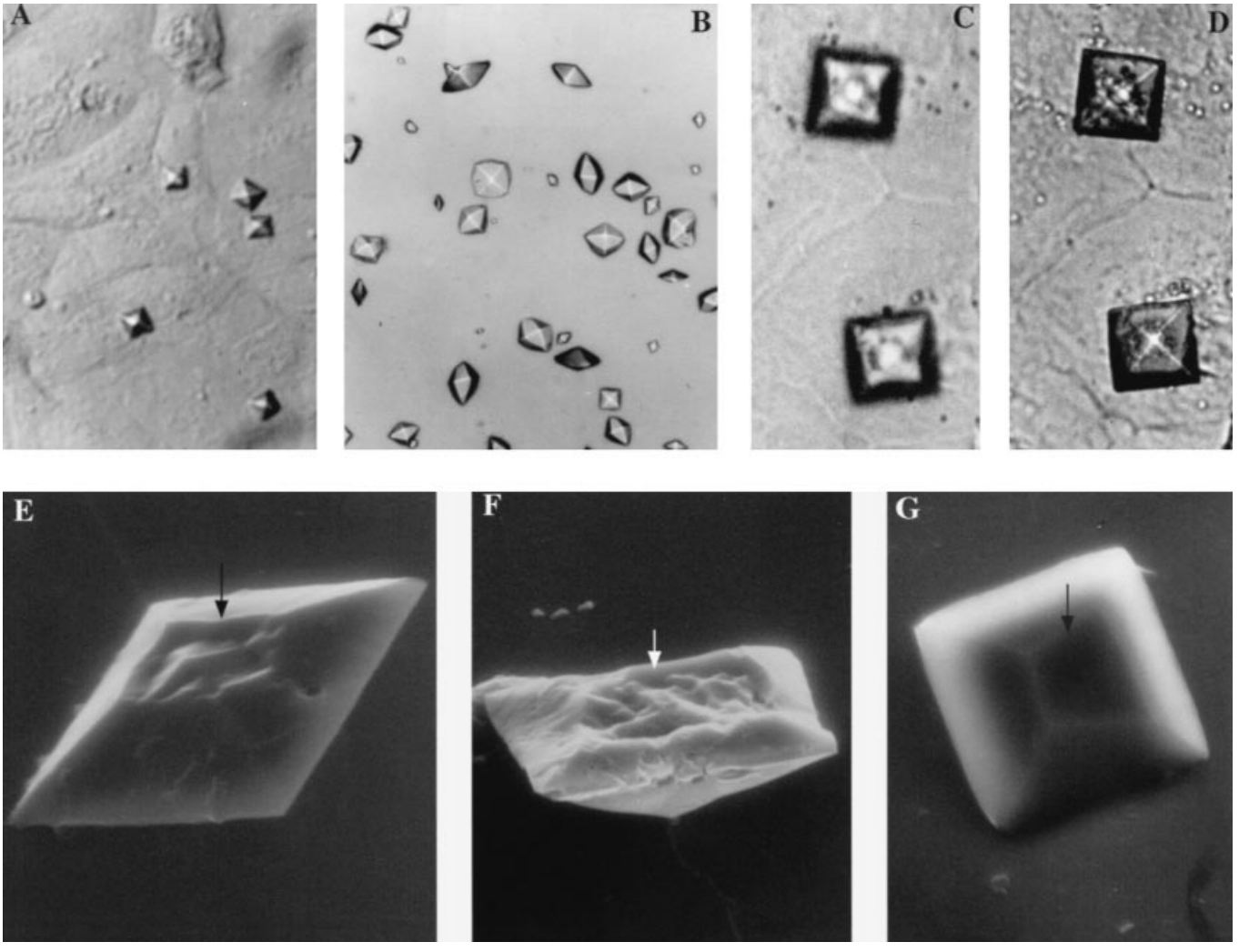

Fig. 2. Interface of COD crystals and the apical plasma membrane of BSC-1 cells. By 12 hours, numerous well-formed COD crystals with uniform morphology were observed on the surface of BSC-1 cells by phase-contrast microscopy (*A*, ×1200). In contrast, crystals nucleated in a random orientation in the absence of cells (*B*, ×1200). To observe the face of contact between cell and crystal, samples were fixed, air dried and carbon coated. When examined under light microscopy, carbon coating partially blocked transmitted light and allowed observation of the face of contact when the focal plane was set at the interface between the crystal and cell (*C*, ×2400). When the focal plane was adjusted above the monolayer, the typical bypyramidal habitus of COD was seen (*D*, ×2400). To observe the underside of crystals nucleated onto the cells using SEM, crystals were removed from the monolayer with a pipette, inverted, placed on a coverslip, air dried, and carbon coated. The flat undersurface seen by light microscopy in Panel C which represents the (001) of COD was confirmed; disturbances of the surface are readily apparent (arrows) (*E*, ×2500; *F*, ×3000). The (001) face does not ordinarily develop during nucleation and growth of COD crystals, but was occasionally observed as a smooth flat surface when COD crystals were grown on a plastic dish in the absence of cells (arrow, *G*, ×3200).

an inanimate surface (Fig. 2B). To identify this face, cells were grown on uncoated glass coverslips in the inner four wells of 24-well tissue culture dishes. Then crystals were nucleated onto the surface of the monolayer as described above. Samples were fixed, dried, coated with carbon and then studied both by transmitted light and SEM. Carbon-coating partially blocked transmitted light and facilitated observation of the contact plane between cell and crystal when viewed through the monolayer (Fig. 2C). If the focal plane was adjusted slightly upwards, the familiar bypyramidal organization of the (101) face and its symmetry-related counterparts was readily apparent (Fig. 2D). The cell-crystal interface was exposed by aspirating a crystal with a pipette, and gently placing it upside down on a coverslip. SEM revealed that crystal-cell contact took place via the (001) face (arrows, Fig. 2 E, F). More than 95% of crystals grew with this common morphology (Fig. 2A) dictated by development of the (100) and (101) faces, and their symmetry-related counterparts, over the (001) basal plane that consistently served as the interface with the cell. As time went by the (001) became progressively disturbed as crystal growth proceeded (Figures 2 E, F). This etching of the

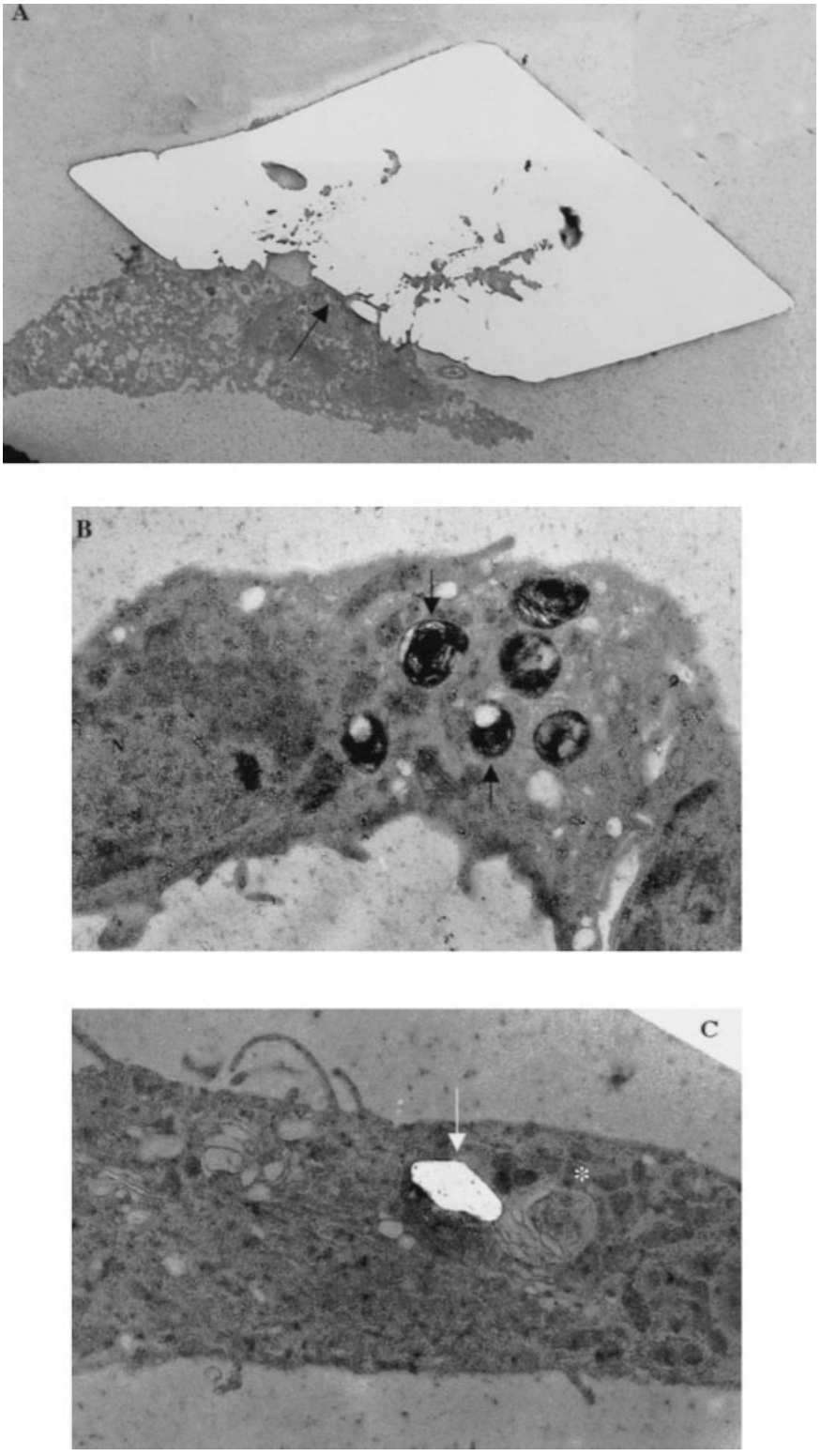

Fig. 3. Transmission electron microscopy (TEM) of COD crystal growth and internalization. To observe COD crystal internalization after nucleation, cell pellets were prepared by scraping cells into an Eppendorf tube, fixing them with half-strength Karnofsky's solution, dehydrating, embedding, and then sectioning for TEM. The disturbed underside [edge of the (001) plane] of a large extracellular COD crystal grown for 12 hours is seen (arrow), providing evidence of an active cellular response during nucleation and growth (*A*, ×6500), although the cell was disrupted during preparation for study. Numerous intracellular myeloid bodies (arrows) were observed by six hours of growth (*B*, ×30,000), possibly the result of ongoing crystal internalization and dissolution. Additional smaller crystals may have been dissolved during fixation and subsequent processing, because apparent crystals covered by plasma membrane and presumably internalized by cells were observed by light and scanning electron microscopy in Figure 1D. Similar myeloid bodies were not observed in control cells exposed to buffer without oxalate. By 12 hours, numerous small intracellular crystals were seen (arrow), in some instances in close association with myeloid bodies (asterisk, *C*, ×24,000).

upper structural layers of the (001) is likely to be caused by a dynamic cellular response to the crystal, as seen in Figure 1 D-E.

Crystals that developed on the apical surface of cells were also visualized by TEM (Fig. 3A). Subsequent electron diffraction confirmed that these crystalline domains were indeed COD. The numerous disturbances of the crystal surface at its contact interface with the cell, which is the edge of the (001) plane (Fig. 3A), provide additional evidence of an active cellular response during crystal

nucleation and growth. TEM was also used to investigate intracellular events as COD crystal nucleation proceeded on the cell surface. At six hours, numerous intracellular myeloid bodies were observed containing small remnants of crystalline material (arrows, Fig. 3B); similar myeloid bodies were seen during internalization and dissolution of exogenous preformed COM crystals [23]. By 12 hours, numerous intracellular crystals were detected (arrow, Fig. 3C), many in close association with myeloid bodies (asterisk, Fig. 3C). Therefore, nucleation of crystals on the apical cell surface and internalization of them appear to proceed in tandem. Since oxalate ions can be transported into renal cells from the extracellular fluid [24, 25], some of the crystals observed inside cells could have nucleated there.

DISCUSSION

In this report we describe face-specific nucleation of COD crystals onto the apical surface of renal epithelial cells followed by internalization of the crystals, a heretofore unrecognized physiological event that could result in retention of crystals within the nephron, and possibly kidney stone formation when this process is dysregulated or overwhelmed. Small crystals were visualized on the surface of cells after two hours, and by six hours the unambiguous habitus of COD was confirmed. Nucleation onto cells occurred almost exclusively via the (001) face, one that is only rarely observed when COD crystals nucleate onto inanimate surfaces. Initially, COD crystals were internalized almost as quickly as they formed on the apical cell surface. These results suggest a new model of kidney stone formation, and perhaps other biomineralization events.

Face-specificity governing the interaction between calcium-containing crystals and organic molecules has been explored in other experimental systems. Selective adhesion of frog A6 kidney cells to specific faces of calcium tartrate crystals has been described, as well as arginine-glycine-aspartic acid (RGD)-inhibitable binding of these cells to calcite crystals [8]. In these reports, A6 cells were plated directly onto the crystals so, unlike the present study, the crystal-cell interaction took place at the basolateral rather than the apical cell surface.

Development of the (001) face is an unlikely event in COD [18, 26]. Structural and theoretical considerations predict that its growth is not favored because it is an unstable crystal face [18, 26–28]. Just a single published report describes COD crystals purported to exhibit (001) faces [29], and in this instance the crystals were grown under high pressure. Furthermore, we observed the (001) only very rarely during innumerable COD growth experiments utilizing a variety of substrates and dopants. In these selected cases, crystals exhibiting the (001) always accounted for less than 0.5% of the total crystal population in any single experiment, as was the case under control conditions (absence of cells) in the current study (Fig. 2B). Thus it is noteworthy that the apical surface of BSC-1 cells not only favors nucleation of COD crystals, but that it takes place via the (001) face.

Structurally the (001) face of COD is composed of sets of oxygen atoms, W(1) and W(2) from two crystallographically-independent water molecules, together with calcium atoms. Each of these atoms lie exactly on the plane of the (001) since they have zero height along the *c* axis (Fig. 4A) [22]. This organization, unique to the (001), provides an extensive network of potential bonding sites in a manner reminiscent of that found in the (100) faces (Fig. 4B) [10, 22]. Previously we demonstrated that when preformed, well-developed, large COD crystals (25 to 50 μM) were added to BSC-1 cells, preferential bonding occurred at the (100) and symmetry-related faces of the crystals [10]. Importantly, the preformed COD crystals used in that study did not display (001) faces [10], because this face is not usually observed on COD crystals grown under standard conditions [18, 26]. The oriented nucleation of the (001) face and its symmetry-related counterparts induced in the current study suggests that organic macromolecules on the apical renal cell surface have anionic sites organized according to an atomic motif that stereochemically satisfies the demands imposed by both W(1) and W(2) for hydrogen bonding, and by calcium atoms for completing their eight-fold coordination link with the oxygen ligands (Fig. 4A). Furthermore, each of these bonding requirements needs to be simultaneously satisfied; any disturbance will result in structural instability, which may in part explain the disordered appearance of the (001) face in Figure 2 E and F.

The present report and previous studies [30–32] also provide insight into the nature of crystal-binding sites on the surface of renal epithelial cells because molecules on the plasma membrane that favor nucleation of crystals will simultaneously promote their adhesion. Preformed COM crystals bind to anionic, sialic acid-containing glycoproteins on the renal cell surface [30], a process that can be blocked by soluble anions including citrate [31], specific glycoproteins such as osteopontin, and glycosaminoglycans such as heparan sulfate, each of which are found in tubular fluid [31, 32]. Anionic phospholipids [33] and glycosaminoglycans [7] have also been proposed as cell surface binding sites. COD and COM crystals could each adhere to similar molecules such as glycoproteins [30] on the tubular cell surface, because these two crystals share a number of structural similarities [12].

Aggregation of small crystals suspended in the tubule lumen to form an occlusive mass, or adhesion of microcrystals to lining epithelial cells have each been proposed to mediate intrarenal crystal retention and subsequent formation of a kidney stone. Observations in rats made hyperoxaluric by administration of ethylene glycol are consistent with the possibility that calcium oxalate crystals can nucleate either on the surface of tubular cells or within the lumen [34]. However, it has generally been accepted that calcium oxalate crystals nucleate in luminal fluid, and proposed that these crystals subsequently adhere to cells

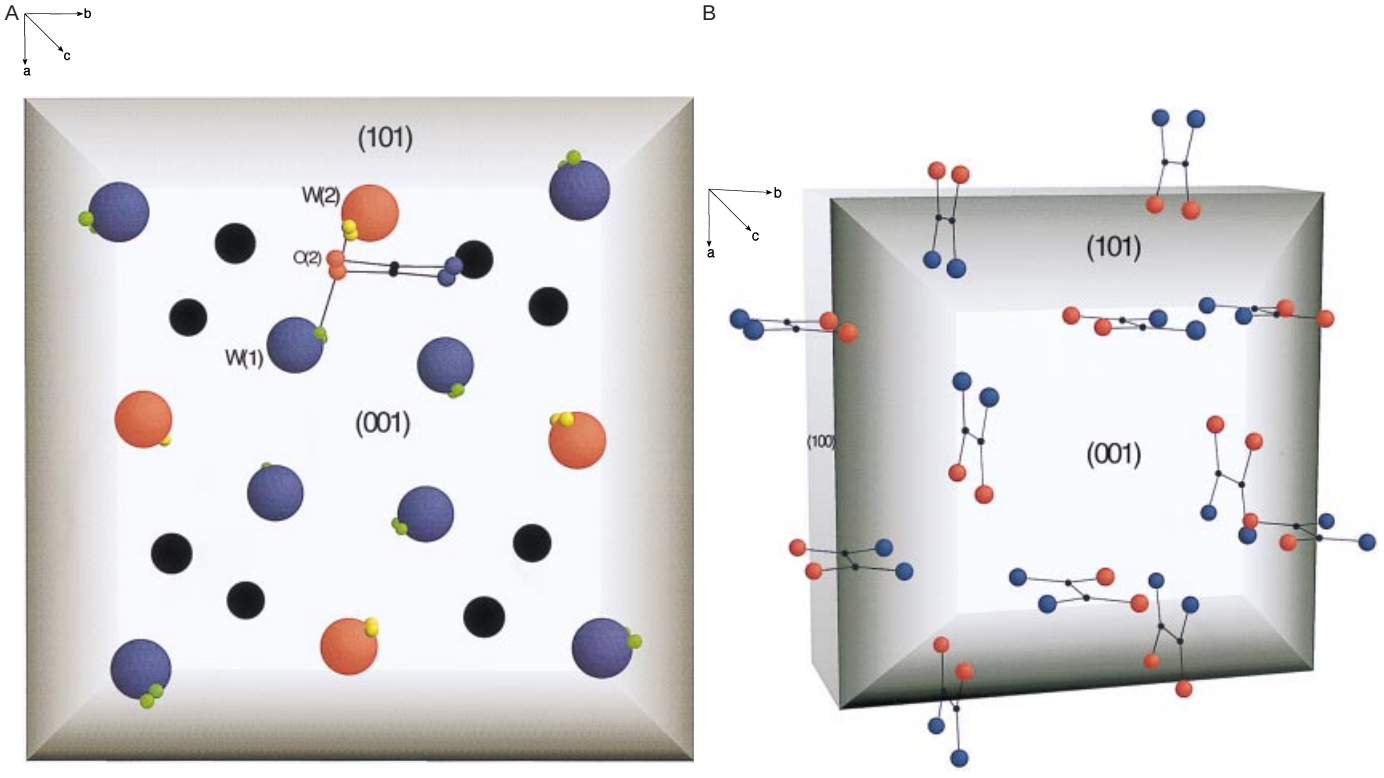

Fig. 4. Partial representation of the atomic structure of COD depicting the availability of oxygen O(2) along the (001) and (100) planes for bonding. (*A*) The (001) face of COD. Black spheres represent calcium atoms, large blue and red spheres are the oxygen atoms from two crystallographically independent water molecules, W(1) and W(2), respectively. All three atoms lie on the (001) plane and have zero height along the *c* axis. Hydrogen bonding between W(1) and W(2) articulates itself through O(2) oxygen atoms (small red spheres) of the carboxylate groups. For clarity, the oxygens of the oxalate groups are depicted as spheres smaller than the oxygens of water molecules. Carbon atoms of the carboxylate groups are small black spheres, and the O(1) oxygen atoms of the carboxylate groups are the small blue spheres. The carboxylate molecules lie above the plane of the (001) and thus provide for both stability of the (001), as well as structural continuity along the c axis. (*B*) Schematic representation of the organization of the carboxylate groups in the structure of COD. Note the orthogonal relationship that the oxalate groups assume with respect to one another. Oxalates extend normally to the highly planar surfaces of the (100) and (001) faces, and their symmetry-related counterparts. O(2), represented by red spheres, is available for hydrogen bonding.

and are then transported into the interstitium by an as yet undefined mechanism [35]. As luminal membrane vesicles can heterogeneously nucleate calcium oxalate crystals, it has been proposed that membrane fragments in tubular fluid, perhaps released by injured cells, could also promote kidney stone formation [36]. The current study suggests a new hypothesis: that the surface of intact, living renal epithelial cells could function as a favored site of crystal nucleation from supersaturated tubular fluid. Once nucleated onto the apical plasma membrane, COD crystals can be internalized by BSC-1 cells (Fig. 1) and subsequently dissolve within lysosomal bodies (Fig. 3) [23]. It is possible that nucleation and internalization of microcrystals on the apical cell surface is an ongoing process in the functioning nephron, that internalization of microcrystals nucleating on the cell surface or in luminal fluid defends the tubule against further growth of crystals anchored to the plasma membrane, and that formation of a kidney stone occurs when these processes are disturbed. For example, an increased number of crystal nucleation sites on the cell surface could favor crystal formation and subsequent crystal growth, as might decreased tubular fluid concentrations of citrate or other anionic inhibitors of crystal nucleation and growth. Alternatively, disordered cell function might prevent or delay internalization of a nucleated crystal that was anchored to the cell surface, thereby allowing its continued growth while bathed in supersaturated tubular fluid. Crystal retention could also result from delayed intracellular dissolution of internalized crystals. Consistent with this hypothesis is the observation that renal injury induced by a nephrotoxic antibiotic (gentamicin), given to rats rendered hyperoxaluric by a dietary manipulation, enhanced kidney stone formation [37]. Once renal epithelial cells internalize crystals, a cascade of responses can ensue including altered gene expression [38], cytoskeletal reorganization [5], and proliferation [3]. The contributions of these cellular responses to kidney stone formation remain to be determined.

In conclusion, COD crystals can nucleate in a stereospecific manner onto the surface of living renal cells and

subsequently be internalized by them. Crystal nucleation onto the apical plasma membrane of tubular epithelial cells *in vivo*, followed by internalization, could be an important, hitherto unrecognized physiological phenomenon in the mammalian kidney that under pathological conditions results in stone formation. Nucleation of other types of crystals onto cell surfaces could mediate mineral deposition in diverse disease states including arthritis [14] and atherosclerosis [13], as well as during normal development of bones and teeth [17], and the formation of invertebrate exoskeletons [15].

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APPENDIX

Abbreviations used in this article are: BSC-1, monkey renal epithelial cells; COD, calcium oxalate dihydrate; COM, calcium oxalate monohydrate; DMEM, Dulbecco's modified Eagle's medium; EDS, energy dispersive mode in x-ray microanalysis; MDCK, Madin-Darby canine kidney cells; RGD, arginine-glycine aspartic acid; SEM, scanning electron microscopy.

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