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Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals

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Abstract The reaction of Madin-Darby canine kidney cells (MDCK) to potassium oxalate (KOx), calcium oxalate monohydrate (COM) crystals, or a combination of the two was studied. The most noticeable effect of exposure of the cells to either KOx or COM crystals was loss of cells from the monolayer ranging from 20% to 30%, depending upon the particular treatment. Cellular enzyme values in the media were elevated significantly by 12 h of exposure, although in specific instances, elevated levels occurred at earlier time periods. As regards the monolayer, trypan blue exclusion was decreased significantly, although amounting to only a 4–5% reduction. Specific tritiated release occurred at 4 and 12 h after exposure to KOx and at 12 h after exposure to crystals. Structurally, COM-cell interactions were complex and extensive endocytosis was noted. Cells were released from culture either as cell-crystal complexes or from the intercellular spaces after exocytosis. When treatments were combined the effects were only slightly additive, but the two treatments potentiated each other: all media enzyme levels (with one exception) were elevated at 2 h, tritiated adenine release was present at 4 h, and there was more extensive cell loss from the culture monolayer. These data suggest that both KOx and COM crystals damage MDCK cells when applied alone, and in concert they act synergistically.

Key words MDCK · Calcium oxalate · Oxalate · Scanning Electron microscopy · Trypan blue · Adenine

Experimentally, formation of calcium oxalate (CaOx) crystals in renal tubular lumens occurs with intimate contact between the crystals and cellular components, especially luminal membranes [13]. In a sub-lithogenic hyperoxaluric rat model the addition of a cell membrane shedding nephrotoxin initiated CaOx formation, suggesting that the presence of membranes in a metastable CaOx environment stimulates heterogeneous nucleation [10]. In a separate series of experiments in vivo, induction of mild hyperoxaluria resulted in low-grade tubular cell damage, as demonstrated by enzymuria, without apparent crystal formation [15]. Additional evidence that cells contribute to crystal formation comes from experiments in vitro in which isolated cell membranes and membrane phospholipids support CaOx crystallization in metastable CaOx solutions [14, 16]. While previous work of this type implicates a role for cell injury in stone formation, evidence from such in vivo experiments is somewhat indirect and the question as to whether or not oxalate (Ox) or CaOx crystals can themselves cause cell damage is difficult to determine. We asked whether the Ox ion is toxic to cells, whether CaOx crystals can damage cells, and whether the effects of the two agents are additive. In order to explore this question, we examined the effects of Ox ion at a low level of relative supersaturation (RSS) in the culture medium, COM crystals and combined Ox/COM upon a cell culture line. We employed cultures of MDCK (Madin-Darby canine kidney cells), a cell line having many of the characteristics of mammalian cortical collecting tubules [29]. Cells were grown on membrane supports and were examined for cell morphology, cell viability, and enzyme alterations including the cell membrane enzymes γ-glutamyl transpeptidase (GGT) and leucine aminopeptidase (LAP), the lysosomal enzyme *N*-acetyl-β-glucosaminidase (NAG), and the cytosolic enzyme lactate dehydrogenase (LDH).

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Materials and methods

Cell culture

For routine maintenance, MDCK cells (ATCC CCL 34, Rockville, Md) passages 53–63 were maintained as sub-confluent monolayers on 75 cm^2 Flacon T-flasks in 50:50 Dulbecco's modified essential

medium nutrient mixture F-12 (D-MEM/F-12) containing 15 mmol/l HEPES, 10% fetal calf serum, streptomycin (0.20 mg/ml) and penicillin (1.0×10^2 IU/ml) pH 7.40, at 37°C in a 5% CO_2 air atmosphere [9]. Medium was replenished two or three times weekly. Cells were subcultured by disassociation with 0.05% Trypsin and 0.53 mmol/l ethylenediaminetetraacetic acid (EDTA).

Oxalate studies

Cells for experiments were seeded onto 25 mm Falcon membrane inserts. Semi-confluent monolayers were weaned from serum to a defined D-MEM/F-12 medium supplemented with insulin, transferrin, ethanolamine, selenium, hydrocortisone, prostaglandin E_1 and 3,3',5-triiodo-L-thyronine (T_3) [28]. Oxalate as KOx was added using a stock solution of 10 mmol KOx in normal sterile saline passed through filters with pore size 0.2 µm. In order to avoid spontaneous nucleation in the media, KOx was diluted to a final concentration of 0.25 mmol/l, a concentration found in normal human voided urine and at which no crystallization occurred in the culture medium. This level of Ox results in a CaOx RSS of 3.6 in the media. Cells were incubated with KOx for 2, 4 and 12 h. Samples were run in triplicate in two separate experiments.

Crystal preparation

COM was prepared by adding equal, unbuffered volumes of 1 mmol/l $CaCl_2$ and 10 mmol/l $K_2C_2O_4$ together at room temperature. After 10 min, suspension was filtered onto a 0.2-µm filter, washed, and stored desiccated [1]. Scanning electron microscopy (SEM) examination verified an average crystal size of 0.6 µm. Crystals analyzed before and after UV sterilization and equilibration in defined medium were confirmed to be pure COM by high-resolution X-ray power diffraction using a Philips APD 3720.

Seed slurry preparation and addition to cultures

UV-sterilized crystals were equilibrated in defined medium at a concentration of 10 mg/ml, at 37°C 24 h before use and added to individual inserts at a final concentration of 500 µg/ml. Cells were handled as in the KOx experiments: monolayers were incubated with crystals for 2, 4 and 12 h. In a third set of experiments, combined effects were studied by incubating culture cells with solutions containing both KOx and COM in the concentrations noted above, for 2, 4, and 12 h.

Cell viability

The inability of cells to exclude trypan blue was used to evaluate cell damage. Isotonic 0.4% trypan blue was added to trypsinized, single cell suspensions and counted on a hemocytometer [7]. Total cell number counts from the monolayer were made on this same aliquot. In parallel studies cells were labeled with ^3H adenine, and specific release of adenine into the culture medium was calculated as an indicator of sub-lethal cell toxicity. Confluent cells on membranes were radiolabelled with 1 µCi/well of ^3H adenine for 12–16 h. After labelling, the cells were washed four times with Hanks' balanced salt solution (HBSS) to remove excess label and subsequently treated with experimental agents. At the conclusion of the experiment supernatants were retained and spun to remove debris, monolayers were washed four times with HBSS, and washes were pooled. Cells were then lysed with 2% Triton X-100. Specific adenine release was expressed as follows: $((A - C) \div (B - C)) \times 100$, where A = d.p.m. in the supernatant and washes of treated cells, B = d.p.m. in the supernatant, washes and cell lysates of treated cells, and C = the d.p.m. in the supernatant and washes of untreated (control) cells. Spontaneous release was approximately 1% per hour for control cells [2].

Enzyme analysis

Spent media from individual wells were recovered and centrifuged to remove crystals and cellular debris. Processed media was analyzed for various enzyme activities. NAG (EC 3.2.1.30) was measured as described by Kornfeld and Maruhn [17, 21], GGT (EC 2.3.2.2) was determined according to Naftalin [23], LAP (EC 3.4.1.1) was measured according to Bergmeyer [4], and LDH (EC 1.1.1.27) was assayed as described by Vassault [30]. Enzyme activity was expressed as IU/ml media.

Scanning electron microscopy

Membrane-grown cells, incubated as described above, were fixed in 3% glutaraldehyde, buffered by 0.1 mol/l cacodylate buffer and 0.1 mol/l sucrose, for 15 min. After washing, post fixation was with buffered 1% OsO_4 for 10 min. Cells were dehydrated through a graded ethanol series at 10-min intervals and dried using hexamethyldisilazane (HMDS) [24]. Specimens mounted with graphite paint were coated with silver and examined with a JEOL JSM 35C.

Transmission electron microscopy (TEM)

Specimens were treated as for SEM, except for those inserts exposed to COM crystals. After fixation monolayers with crystals were treated with 0.1 mol/l EDTA in the cacodylate/sucrose buffer for 15 min to dissolve the crystalline matter. After washing, specimens were osmicated, washed, dehydrated through graded alcohols, and infiltrated with Spurr's resin [12]. Ultrathin sections were post-stained with uranyl acetate and lead citrate and examined on a ZEISS 10L/LR.

Light microscopy (LM)

Inserts were fixed as described above, dehydrated in graded alcohols through xylene, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Statistical analysis

Significance of enzyme activities, specific release of adenine, and uptake of trypan blue were analyzed for multiple comparison of means using SAS software.

Results

KOx studies

Exposure of MDCK cells to 0.25 mmol/l KOx resulted in an approximate 24% loss of cells from the monolayer as compared to control cultures. This effect occurred within the first 2 h, and similar but smaller losses occurred at 4 and 12 h. Of the cells remaining in culture, a small but significant percentage demonstrated damage: at 2, 4, and 12 h, approximately 4% of the cells remaining on the insert excluded trypan blue (Table 1). Tritiated adenine release demonstrated no change at 2 h, but significant increases in release occurred at 4 and 12 h (Table 1). Enzyme levels in the media demonstrated no significant alteration in LDH until 12 h of exposure. Progressive elevation in NAG levels reached statistical significance after 12 h of exposure. The

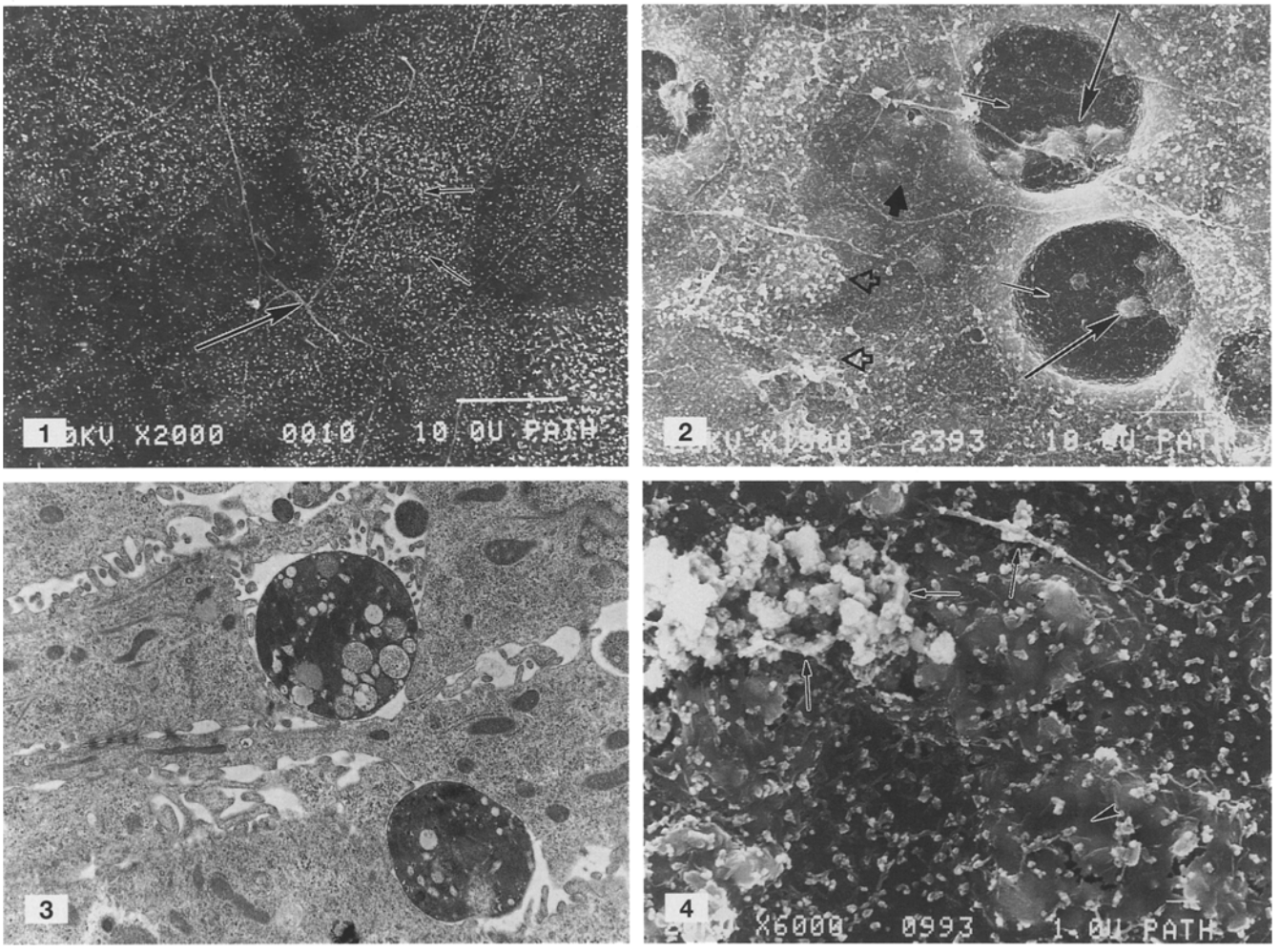


Fig. 1 Scanning electron microscopy (SEM) of control Madin-Darby canine kidney (MDCK) cells. Larger cells with discrete cell-to-cell contacts are present, their surfaces covered with uniform microvilli (*small arrows*). Long tendrils of collapsed cilia are present (*large arrow*)

Fig. 2 SEM of cells exposed to 0.25 mmol/l potassium oxalate (KOx) for 2 h. Note the gaps where cells have been lost from the culture plate (*small arrows*). Residual cell remnants are also shown (*large arrows*). The adjacent cells are distorted with loss of surface microvilli (*solid arrowhead*), and focal blebbing of remaining microvilli (*empty arrowheads*)

Fig. 3 Transmission electron microscopy (TEM) of cells exposed to 0.25 mmol/l KOx for 4 h; tangential section. In the intercellular spaces, darkly staining cytoplasmic debris containing lighter staining areas and lipid droplets are identified

Fig. 4 SEM of cells exposed to calcium oxalate monohydrate (COM) crystals for 2 h. The crystals are tangled with microvilli and cilia (*small arrows*). Focal areas of cell surface contain no microvilli or cilia (*arrowhead*)

Table 1 Specific release of ^{3}H-Adenine trypan blue dye exclusion, and recovery of cell numbers from Madin-Darby canine kidney (MDCK) cells exposed to potassium oxalate (KOx). *P*-values refer to differences from corresponding control values. Mean ± SD

Group ($n=12$)	^{3}H-Adenine release[a]	Trypan blue exclusion[b]	Cell number $\times 10^{6}$[c]
Control	–	99.2 ± 0.1	2.81 ± 0.15
KOX: 2 h	−1.63 ± 1.95	95.3 ± 0.3 $P<0.001$	2.14 ± 0.12 $P<0.001$
KOx: 4 h	7.05 ± 1.66 $P<0.001$	94.3 ± 0.3 $P<0.001$	2.30 ± 0.10 $P<0.001$
KOx: 12 h	6.76 ± 2.01 $P<0.001$	93.9 ± 0.3 $P<0.001$	2.34 ± 0.13 $P<0.001$

[a] Percent specific release over control cells
[b] Percent of cells remaining in culture that excluded the dye
[c] Cells recovered in suspension after EDTA/Trypsin treatment

Table 2 Media enzyme activities (IU/ml) of MDCK cells exposed to KOx (*LDH* lactate dehydrogenase; *NAG* *N*-acetyl-β-glucosaminidase; *LAP* leucine aminopeptidase; *GGT* γ-glutamyl transpeptidase)

Group ($n=12$)	Media LDH	Media NAG	Media LAP	Media GGT
Control	25 ± 6	0.47 ± 0.04	0.21 ± 0.11	0.60 ± 0.13
KOx: 2 h	23 ± 8	0.61 ± 0.09	0.80 ± 0.25	1.33 ± 0.34 $P<0.05$
KOx: 4 h	29 ± 5	0.49 ± 0.05	1.13 ± 0.38 $P<0.02$	1.01 ± 0.32
KOx: 12 h	45 ± 9 $P<0.01$	0.83 ± 0.08 $P<0.001$	1.17 ± 0.31 $P<0.02$	2.91 ± 0.29 $P<0.001$

Table 3 Specific release of ^{3}H-adenine trypan blue dye exclusion, and recovery of cell numbers from MDCK cells exposed to calcium oxalate monohydrate (COM) crystals (units and terms as in Table 1)

Group ($n=12$)	^{3}H-adenine release	Trypan blue exclusion	Cell number $\times 10^6$
Control	-	99.2 ± 0.2	2.17 ± 0.10
COM: 2 h	-2.87 ± 1.97	95.3 ± 0.5 $P<0.001$	1.48 ± 0.05 $P<0.001$
COM: 4 h	-3.55 ± 3.90	95.6 ± 0.3 $P<0.001$	1.49 ± 0.08 $P<0.001$
COM: 12 h	7.31 ± 2.66 $P<0.05$	96.5 ± 0.1 $P<0.001$	1.42 ± 0.06 $P<0.001$

Table 4 Media enzyme activities of MDCK cells exposed to COM crystals

Group	Media LDH	Media NAG	Media LAP	Media GGT
Control ($n=10$)	5.0 ± 0.9	0.03 ± 0.03	0.52 ± 0.08	0.52 ± 0.11
COM: 2 h ($n=9$)	8.0 ± 1.7	0.13 ± 0.03	2.02 ± 0.35 $P<0.002$	0.22 ± 0.17
COM: 4 h ($n=9$)	8.0 ± 1.0	0.36 ± 0.03 $P<0.01$	1.33 ± 0.15 $P<0.001$	0.57 ± 0.20
COM: 12 h ($n=9$)	20.0 ± 2.5 $P<0.001$	0.37 ± 0.02 $P<0.001$	2.41 ± 0.22 $P<0.001$	1.16 ± 0.17 $P<0.001$

Table 5 Specific release of ^{3}H-adenine trypan blue dye exclusion, and recovery of cell numbers from MDCK Cells exposed to COM crystals and KOx (units and terms as in Table 1)

Group ($n=12$)	^{3}H-Adenine release[a]	Trypan blue exclusion[b]	Cell number $\times 10^{6c}$
Control	-	97.9 ± 0.3	1.58 ± 0.05
COM + KOx: 2 h	1.54 ± 2.16	92.4 ± 0.4 $P<0.001$	0.90 ± 0.03 $P<0.001$
COM + KOx: 4 h	5.86 ± 3.48 $P<0.01$	92.1 ± 0.4 $P<0.001$	1.04 ± 0.02 $P<0.001$
COM + KOx: 12 h	15.58 ± 3.30 $P<0.001$	91.8 ± 0.4 $P<0.001$	0.92 ± 0.04 $P<0.001$

Table 6 Media enzyme activities of MDCK cells exposed to COM crystals and KOx

Group ($n=12$)	Media LDH	Media NAG	Media LAP	Media GGT
Control	5.7 ± 0.9	0.05 ± 0.01	0.12 ± 0.01	1.42 ± 0.04
COM + KOx: 2 h	16.9 ± 2.56 $P<0.001$	0.16 ± 0.01 $P<0.001$	0.23 ± 0.03 $P<0.01$	1.50 ± 0.06
COM + KOx: 4 h	21.4 ± 3.0 $P<0.001$	0.20 ± 0.01 $P<0.001$	0.37 ± 0.03 $P<0.001$	1.74 ± 0.05 $P<0.001$
COM + KOx: 12 h	20.9 ± 2.1 $P<0.001$	0.32 ± 0.02 $P<0.001$	1.60 ± 0.08 $P<0.001$	2.42 ± 0.25 $P<0.001$

LAP and GGT demonstrated progressive increases in release, achieving significance at 4 and 12 h (Table 2).

Compared with control cultures (Fig. 1), examination of the experimental cultures with SEM revealed comparable changes at all time periods. Where cell loss from the culture had occurred large gaps were present and adjacent cells demonstrated either loss of microvilli or swelling and blebbing of the remaining microvilli (Fig. 2). With TEM no specific effects were noted, but focal cell damage was present, as demonstrated by the presence of densely staining bodies in the intercellular spaces (Fig. 3).

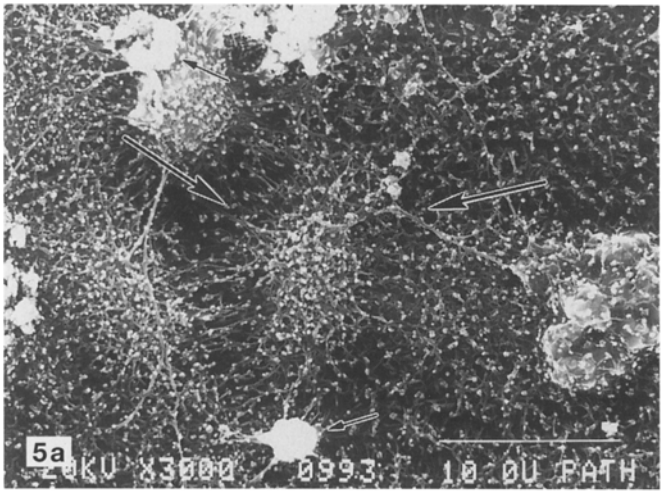
COM studies

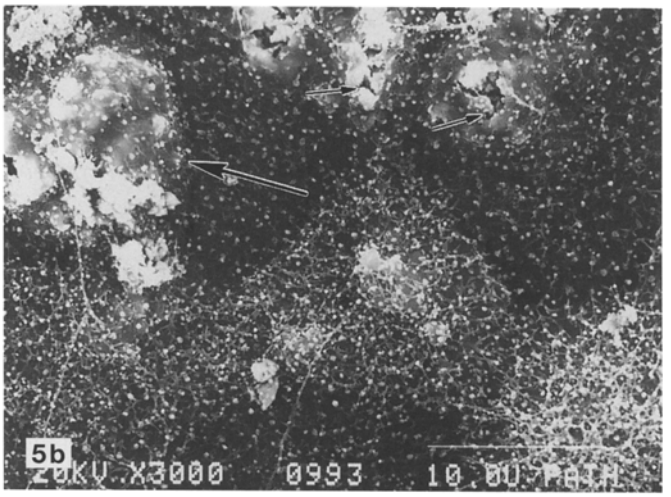
After exposure to COM crystals for 2 h, approximately 32% of cells were lost. Because of cell-crystal aggregates, exact numbers of cells lost were difficult to determine and the magnitude of loss is probably overestimated. The initial effect was present at the 2 h point and persisted at 4 and 12 h. The remaining cells in culture showed small but significant changes in viability; approximately 4% of the cells were unable to exclude trypan blue at all time periods. Tritiated adenine release, however, did not reach significance until 12 h of exposure (Table 3). Enzyme activity in the media showed a progressive increase with time, reaching significance with NAG at 4 h of exposure and with LDH and GGT at 12 h of exposure. By contrast, media LAP achieved significant increases in activity by 2 h and this persisted at the 4 and 12 h time periods (Table 4).

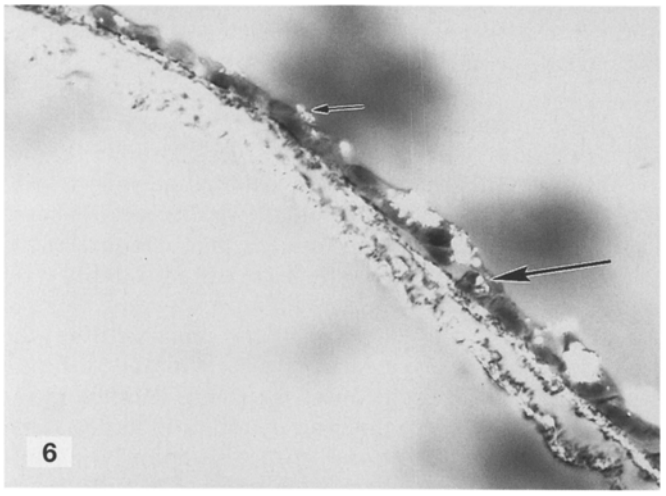
Examination of the culture inserts with SEM revealed a complex set of morphological alterations observed within 2 h with similar but more extensive changes present at 4 and 12 h. Contact between crystals and cells at 2 h resulted in intricate formations in which the crystals became enmeshed with microvilli and cilia of the cell surfaces (Fig. 4). Large patches of adjacent cell surfaces were cleared of microvilli. Subsequently, individual cell-crystal complexes appeared to separate from adjacent cells, resulting in elongated and stretched portions of adjacent cell membrane and cell-to-cell contacts (Fig. 5a). Also, large aggregates of crystals appeared to protrude from underneath the culture, commonly, but not always, at the cell-to-cell interface (Fgi. 5b). LM examination of cells exposed to COM revealed crystals attached to the surface of the cells and extensively endocytosed (Fig. 6). TEM of similar cultures revealed endocytosis of crystals, visualized as crystal ghosts, either as small crystals or large agglomerates. Crystals were present within the cytoplasm or extruded into the lateral intercellular spaces, in a similar way to that shown in Fig. 5b. In these spaces, crystals and flanking cytoplasmic membranes formed complex aggregates (Fig. 7).

Combined studies with KOx and COM

Exposure of cultures to both KOx and COM crystals resulted in an approximate 40% loss of cells at each of the time periods studied, and about 5% of the remaining cells







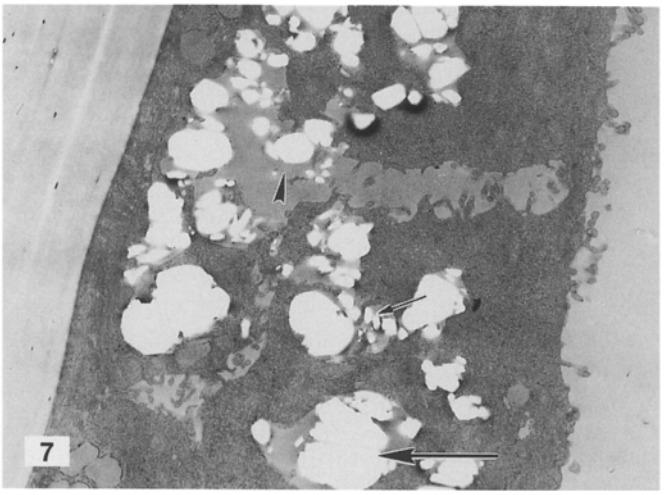


Fig. 5 a SEM of MDCK cells exposed to COM crystals for 2 h. Crystal-cell complexes are present, which appear to be separating from the culture (*small arrows*) with stretching of cell-to-cell contacts (*large arrows*). **b** Crystals are present underneath cells (*small arrows*) and where they appear to be leaving the plate at cell-to-cell contacts (*large arrow*)

Fig. 6 Light microscopy of cells exposed COM crystals for 2 h. Crystals are attached to cell surfaces (*small arrow*) and others have been endocytosed (*large arrow*)

Fig. 7 TEM of MDCK cells exposed to COM crystals for 12 h. Crystals (seen as ghosts) were endocytosed regardless of size as smaller forms (*small arrow*) or larger agglomerates (*large arrow*). Crystals are shown being released into the intercellular space (*arrowhead*) where they form crystal-cell surface complexes

in culture were unable to exclude trypan blue. Tritiated adenine release showed no change at 2 h, but a significant release at both 4 and 12 h (Table 5). A progressive increase in the media activity of all four enzymes occurred and significance was achieved at 2 h with LDH, NAG, and LAP, and at 4 h with all four enzymes studied (Table 6). On SEM, the changes illustrated were similar to those described for exposure to COM crystals, and no difference in the quality of the changes was noted with the combined exposure.

Discussion

These experiments establish that exposure of MDCK cells to a low concentration of KOx has a demonstrable effect upon the monolayer. The greatest impact of this effect was exhibited by loss of cells from the cultures. The morphological studies suggest alterations in cell-to-cell contacts in areas of cell loss and decreased adherence of the cells to the substrate. In addition, a small but significant decrease in viability occurred in the remaining monolayer cells. The process occurs rapidly within the time-frame of this work, in that these effects were already evident after treatment for 2 h. No noticeable differences in values were found at 4 and 12 h. Increased adenine release indicative of sub-lethal injury was also shown, but not until after 4 h and 12 h of treatment. Although media enzyme concentrations increased with all four enzymes measured by 12 h, the two membrane-associated enzymes, GGT and LAP, appeared

to be more sensitive to Ox; there were significant changes at 2 and 4 h, respectively. These data suggest that a subset of cells within the culture is sensitive to Ox, since replacement of damaged cells within 2 h is unlikely. The biochemical indicators for cell damage were supported by morphological studies, as profound cellular alterations were demonstrated by SEM and TEM.

Summation of these findings support the contention that exposure of MDCK cells to Ox results in cell damage [11]. These results are meaningful because the MDCK cell is relatively resistant to injury, at least compared with cells of proximal tubular origin [3, 26]. Other studies more directed towards oxalate-cell interactions and their relationship to nephrolithiasis have concentrated upon membrane transport processes. Gambaro and Baggio [8] postulated that idiopathic calcium nephrolithiasis is a generalized cellular anomaly, as adduced by experiments with red blood cells from stone formers. Data from their work suggest that Ox exchange occurs more rapidly in stone formers and is associated with a more rapid intestinal absorption of Ox. In another series of investigations exploring the interaction of Ox with renal tubular cells in primary culture, Sigmon et al. [27] demonstrated that Ox uptake was significantly greater in renal papillary cells from stone-forming animals than in cells from controls. Oxalate was shown to form complexes with intracellular Ca in renal papillary cells, suggesting that CaOx crystals may be formed or retained by such a mechanism [31]. In another contribution from this group, it was demonstrated that exposure of LLC-PK_1 cells to Ox concentrations from 0.1 to 2 mmol/l resulted in cell damage [22]. Although the mechanisms involved are unclear at this time, the demonstration that cell injury results from continued exposure to Ox supports a hypothesis [11, 14] that in cell-injury-mediated CaOx crystalluria, Ox itself could serve as the primary agent of injury.

The indicators for cell injury in the COM studies gave results similar to those with Ox. As might be anticipated, there were major structural differences, which suggests that while the levels of injury were similar in both experiments, the mechanisms probably differ. Crystals of COM were actively endocytosed, individually or as aggregates. The crystals were intermingled with microvilli and cilia at the surface, as demonstrated by SEM, and adjacent cells were structurally altered. The cell-crystal complexes appeared to separate from the monolayer by one of two mechanisms. In the first, as visualized in Fig. 5a, the crystals and cells or parts of cells were lost from the insert as a unit prior to endocytosis. In the second, crystalline aggregates were extruding from beneath the cell layer, usually at cell-to-cell contact regions, where large gaps could be observed. This interpretation was supported by TEM findings that endocytosed crystals were exocytosed into lateral intercellular spaces accompanied by gaps in those spaces. In these circumstances, the crystals were usually present as complexes of crytals and portions of adherent cellular material.

Interactions between CaOx crystals and cells have been examined by a number of investigators. Seminal work by Mandel and Riese [20, 25] gave qualitative and quantitative evidence that binding specificity exists when renal papillary cells in culture are exposed to COM crystals. Binding sites were selectively located in cell clumps that had lost their attachment to the substrate, suggesting that loss of such contact exposed membrane molecules from the basolateral or basal regions, permitting crystal attachment. Other researches tested the effects of COM crystals on several cell lines, including MDCK cells, and found that exposure induced cell proliferation in the renal cell lines, but not fibroblasts, again indicating an specific interaction between CaOx crystals and renal epithelial cells. On LM and EM, COM crystals were found to be endocytosed by BSC-1 renal cells; similar experiments using MDCK cells were described but not illustrated [18, 19]. However, phagocytosis of urate crystals by MDCK cells has been described [6]. Specificity of COM binding to MDCK cells is inhibited by heparin and sodium pentosan sulphate [5], which supports the observations of Mandel and Lieske.

The importance of the current work is demonstrated by the results of exposing MDCK cells to both Ox and COM crystals. The pattern for tritiated adenine release was similar to that with Ox, but at 12 h the level of release was higher in the combined studies than in either of the other two experiments. While decreases in viability were small in all experiments, the alterations were greater in the combined studies. Even more obvious were the increased levels in the media enzymes. With the exception of GGT, media enzyme levels were considerably higher with the combined studies and more, more importantly, were demonstrated to occur at the 2-h point, as against later points in the other two studies. This effect was particularly striking with LDH, the release of which is a generally recognized indicator of cell injury. Although the combined effects of Ox and COM upon MDCK cells are probably slightly additive, the two agents undoubtedly act synergistically. While the current work demonstrates that Ox or COM acting alone results in cell damage, the synergistic effects of Ox *and* COM treatments are salient and suggest that experiments studying the interaction between crystals and cells should be performed in an Ox environment, especially since COM crystals in the intact organism do not exist in the absence of Ox.

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