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INVITED REVIEW

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Effects of luminal oxalate or calcium oxalate on renal tubular cells in culture

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Abstract Oxalate or calcium oxalate crystal-induced tissue damage could be conducive to renal stone disease. We studied the response of renal proximal (LLC-PK1 and MDCK-II) and collecting (RCCD1 and MDCK-I) tubule cell lines to oxalate ions as well as to calcium oxalate monohydrate (COM) crystals. Cells grown on tissue culture plastic or permeable growth substrates were exposed to high (1 mM) and extremely high (5 and 10 mM) oxalate concentrations, or to a relatively large quantity of crystals (146 μg), after which cell morphology, prostaglandin E_2 (PGE_2) secretion, [^{3}H]thymidine incorporation, total cell numbers and various forms of cell death were studied. Morphological alterations, increased PGE_2 secretion, elevated levels of DNA synthesis and necrotic cell death were induced by extremely high, but not by high oxalate. Crystals were rapidly internalized by proximal tubular cells, which stimulated PGE_2 secretion and DNA synthesis and the release of crystal-containing necrotic cells from the monolayer. Crystals did not bind to, were not taken up by, and did not cause marked responses in collecting tubule cells. These results show that free oxalate is toxic only at supraphysiological concentrations and that calcium oxalate is toxic only to renal tubular cells that usually do not encounter crystals. Based on these results, it is unlikely that oxalate anions or calcium oxalate crystals are responsible for the tissue damage that may precede renal stone formation.

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Introduction

A kidney stone is a collection of crystals that were not excreted with the urine. By far the majority of stones consist mainly of calcium oxalate. Oxalate is extremely poorly soluble in biological fluids. Fortunately, serum oxalate is usually very low (~2 μM). After glomerular filtration, the concentration of oxalate gradually increases in the nephron to reach critical levels of supersaturation somewhere in the renal distal tubule/collecting ducts (~100–500 μM oxalate).

Studies performed on animals [1–4] and in cell culture [5–7] suggested that oxalate not only leads to supersaturation, but that the anion is also nephrotoxic. Since high levels of oxalate are always accompanied with crystalluria under physiological conditions, it is difficult in these studies to differentiate between the toxic effects caused by oxalate and those due to crystals. The present study was performed to study the effects caused by luminal oxalate ions and calcium oxalate (COM) crystals, separately, to cells with properties of the renal proximal and collecting tubules. The results show that oxalate is toxic to both cell types, but only at supraphysiological concentrations (≥5,000 μM), and that calcium oxalate crystals are toxic to proximal tubular cells, but not to collecting tubule cells.

Materials and methods

Cell cultures

Madin-Darby canine kidney (MDCK) cell strains I and II were kindly provided by Dr. G. van Meer (Laboratory for Cell Biology and Histology, AMC, The Netherlands). Porcine renal proximal tubular cells

(LLC-PK_1) cells were obtained from the American Type Culture Collection. Rat cortical collecting $duct_1$ ($RCCD_1$) cells were kindly provided by Dr. M. Blot-Chabaud (INSERM U246, Faculté de Médecine Xavier Bichat, Paris, France) [8]. Experiments were performed with confluent monolayers grown on solid tissue culture plastic or on permeable supports in a two-compartment culture system (transwells; Corning Costar, Badhoevedorp, The Netherlands).

Morphological studies

Cells cultured on plastic dishes were exposed for 6 h to oxalate added in calcium-free physiological saline (0.9% NaCl) or to serum-free DMEM (1.8 mM calcium) and inspected by phase-contrast microscopy coupled to an AxioCam camera scanner (Zeiss, Oberkochen, Germany). Cells cultured on permeable supports were exposed for 24 h to increasing oxalate or to COM crystals, stained with fluorescein isothiocyanate (FITC)-conjugated phalloidin and inspected by confocal laser scanning microscopy (Zeiss LSM 410).

Prostaglandin E_2

To detect whether oxalate or crystals can provoke an inflammatory response, we measured their effect on prostaglandin E_2 (PGE_2) synthesis. Confluent monolayers grown on permeable supports were incubated for 24 h with oxalate or COM crystals added to the apical compartment in calcium-free buffer, while DMEM 10% FCS was added to the basal compartment. The secretion of PGE_2 into the luminal compartment was measured with an enzyme-linked immunoassay (EIA) Kit (Cayman) according to the instructions provided by the manufacture.

DNA synthesis

To determine whether oxalate and/or crystals could induce mitogenic effects in our cells, we measured their effects on thymidine incorporation. Confluent monolayers were incubated for 24 h with oxalate or COM crystals added to the apical compartment in calcium-free buffer, while DMEM 10% FCS was added to the basal compartment. The next day or 1 day later, the cells are pulse-labeled for 3 h with 3.7 kBq/ml [^{3}H]thymidine, washed three times with PBS and the filters counted in a liquid-scintillation counter. The results were expressed in dpm/filter insert.

Apoptosis and necrosis

To assess the nature of oxalate or crystal-induced cell death, we analyzed their effects on annexin V binding

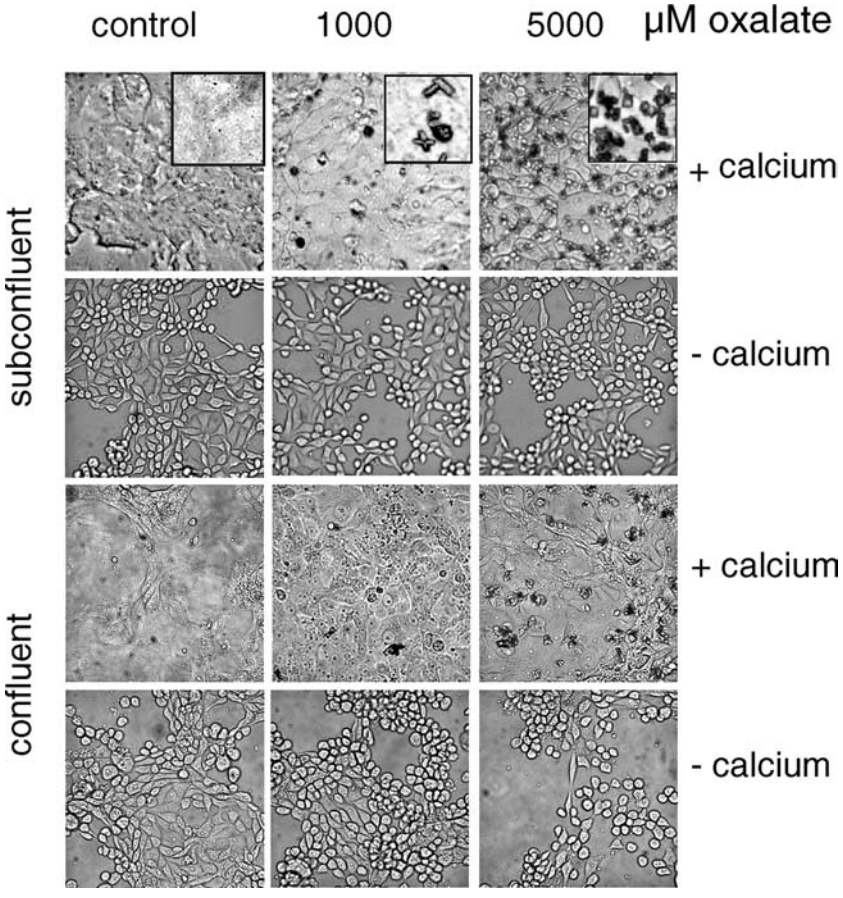

Fig. 1 Subconfluent and confluent LLC-PK1 cultures grown on solid tissue culture plastic and exposed for 6 h to high (1,000 μM) and extremely high (5,000 μM) oxalate added in DMEM (+1.8 mM calcium) or physiological saline (−calcium). Irrespective of the presence of oxalate, cells started to round up and detach from the growth substrate in physiological saline (−calcium). The appearance of the cells in subconfluent and confluent cultures was relatively unaffected in the presence of oxalate added to DMEM (+calcium). Crystals were observed in 1,000 and 5,000 μM oxalate added to DMEM (*see inserts in the top panel*), but not in the dishes where oxalate was added to calcium-free saline. NB Comparable effects were observed in the other cell types exposed to oxalate

and Hoechst 33258 staining (apoptosis), and propidium iodide (PI) influx (necrosis). Confluent monolayers were treated for 24 h with oxalate or COM crystals, washed, and unfixed cells incubated for 15 min with FITC labeled annexin V (1:40) or 0.1 mg/ml Hoechst. The annexin V stained cells were washed three times, fixed in 70% ethanol and incubated for 15 min with 1 μg/ml PI, washed and mounted in Vectashield. Hoechst stained cells were washed, fixed and mounted in Vectashield. Antimycin A (4 h, 1.0 μM) was used as a positive control for apoptosis (annexin V and Hoechst). Treatment of the cells with 70% ethanol served as positive control for necrosis (membrane leakage).

Statistical analysis

All experiments were performed at least three times. The results are presented as means ± SD of three independent inserts. One-way analysis of variance was performed. Differences were considered significant at $P < 0.05$.

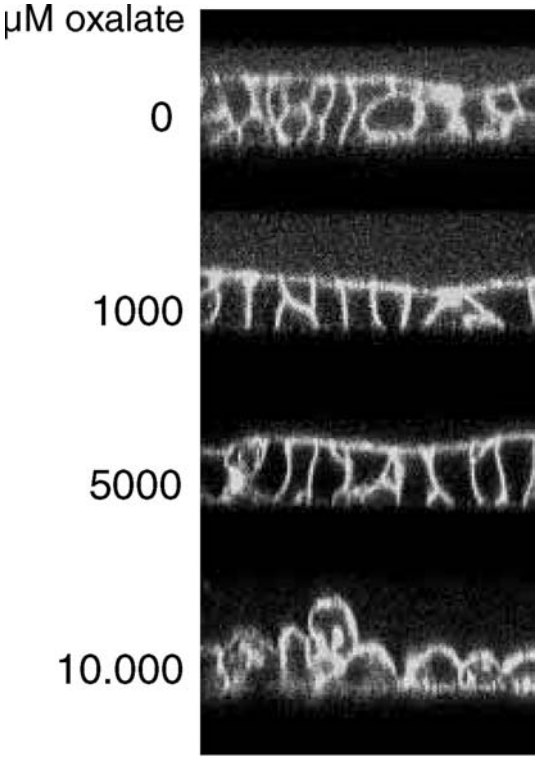

Fig. 2 CLSM images made perpendicular to the growth substrate of confluent MDCK-I monolayers grown on a permeable support in a two-compartment culture system after 24 h incubation with apical 0, 1,000, 5,000 or 10,000 μM oxalate while DMEM 10% FCS was added to the basal compartment. This image shows that the morphological appearance of the cells is not affected by 1,000 or even 5,000 μM oxalate. Only extremely high oxalate (10,000 μM) appears to be lethal to the cells

Results

Morphological studies

Oxalate

Subconfluent and confluent cultures grown on plastic dishes were exposed for 6 h to high (1,000 μM) and extremely high (5,000 μM) oxalate levels added to calcium-containing DMEM (1.8 mM calcium) or to 0.9% NaCl (calcium-free). Phase-contrast microscopy showed that subconfluent and confluent cultures that were not treated with oxalate contained rounded cells under calcium-free conditions. Apparently, the cells started to be released from the growth substrate due to the lack of calcium. The morphological appearance of these rounded cells was not further influenced by high or extremely high oxalate. The exposure of confluent monolayers to high oxalate did not lead to visible morphological effects

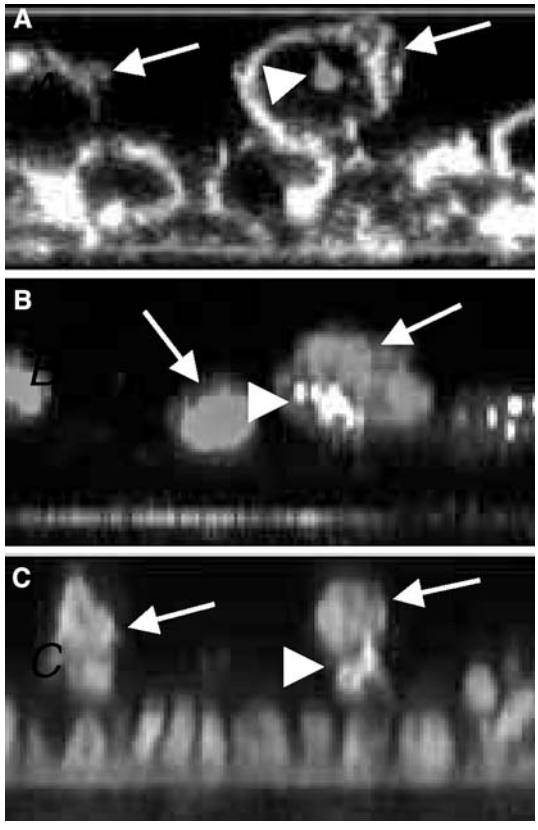

Fig. 3 To study whether crystals can induce physiological (apoptotic), or pathological (necrotic) cell death, MDCK-II cells were incubated for 24 h with COM crystals and subsequently stained with: **a** phalloidine-FITC to visualize the cells, **b** propidium iodide (PI) to study plasma membrane permeabilization (necrosis), or **c** with Hoechst to study DNA fragmentation (apoptosis). The *white triangles* represent crystals reflecting in the laser light, while the *arrows* point at releasing cells. **a** Shows the release of crystal-containing cells from the monolayer, **b** shows that their nuclei are PI positive indicating that they are necrotic. **c** Shows that the DNA in the nuclei of the released cells is not fragmented and that these cells are therefore not apoptotic

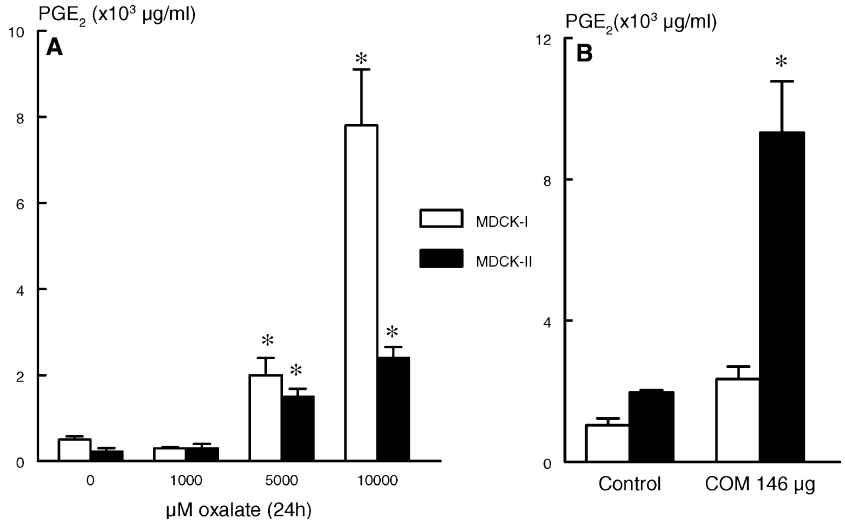

Fig. 4 PGE2 secretion in the apical fluid compartment of confluent renal tubular cell monolayers cultured on permeable supports in a two-compartment culture system and incubated for 24 h with: **a** 0, 1,000, 5,000 or 10,000 µM oxalate, or **b** 0 and 146 µg COM. Oxalate was added to the apical compartment in a calcium-free buffer while the basal compartment received DMEM 10% FCS. Inflammation was induced by very high (5,000 and 10,000 µM), but not by high (1,000 µM) oxalate (**a**). COM provoked a relatively large inflammatory response in MDCK-II, while this reaction was much smaller in MDCK-I (**b**). Means ± SD of a representative experiment are shown. Statistical analysis was performed using ANOVA ($P < 0.05$, $n = 3$). An *asterisk* indicates significantly different compared to untreated controls

in either cell type, while extremely high oxalate resulted in cell rounding (Fig. 1). This effect could be caused by oxalate, but it is also possible that calcium was depleted in the dishes that received extremely high oxalate. Phase-contrast microscopy revealed crystal formation in dishes that received oxalate in DMEM but not in saline. Compared to high oxalate, there were more and smaller crystals formed in dishes that received extremely high oxalate (Fig. 1).

To avoid the influence of crystals in oxalate toxicity studies, these experiments were repeated with cells grown in transwells. In this model system, the cells received oxalate in calcium-free buffer from the apical side, while the basal compartment received DMEM with calcium but no oxalate. Since 6 h oxalate did not cause much effect, we decided to expose the cells to oxalate for a longer period of time. The exposure of proximal or collecting tubule cells on permeable supports for 24 h to high oxalate (1,000 µM) again did not lead to visible morphological alterations. The organization of the cells in the monolayer was disturbed after 1 day of extremely high oxalate (for MDCK-II and LLC-PK1, 5,000 µM and for RCCD1 and MDCK-I, 10,000 µM) (Fig. 2).

Crystals

Next, we studied the morphological response of the various cell types to COM crystals. Crystals rapidly became associated with the plasma membrane of proximal tubular cells to be subsequently taken up by the cells. One day after the addition of crystals, the cells showed a disorderly organization and crystal-containing cells were released from the monolayers (Fig. 3). The incubation of collecting tubular cells with crystals did not lead to crystal binding/internalization. There were no visible morphological alterations in collecting ducts cells incubated with crystals (not shown).

Prostaglandin E2 (PGE_2)

To reveal a possible inflammatory response to oxalate and/or COM crystals, we measured the secretion of PGE_2 in the apical and basal fluid compartment after 24 h exposure to high and extremely high oxalate or COM crystals. $RCCD_1$ and $LLC\text{-}PK_1$ appeared to be less suitable for these studies because they produced hardly detectable levels of PGE_2. Arachidonic acid is converted by cyclooxygenase (COX) isoenzymes to eicosanoids such as PGE_2 and it is known that certain cell lines, including LLC-PK1, have defects in COX activity [9]. These studies were therefore limited to MDCK strains. Relatively high levels of PGE_2 were secreted by both strains during the first days post-seeding; these levels declined when the cultures become confluent (not shown).

Oxalate

Compared to monolayers that did not receive oxalate, PGE_2 secretion was not increased by high oxalate (1,000 µM), while extremely high oxalate (5,000 and 10,000 µM) provoked significantly increased levels of PGE_2 in both MDCK strains (Fig. 3).

Crystals

The secretion of PGE_2 was significantly higher after the incubation of confluent MDCK-II monolayers with COM. PGE_2 secretion was also slightly higher in MDCK-I cells incubated with crystals (Fig. 3).

DNA synthesis and cell counting

To study the effect of oxalate on DNA synthesis, the cells were pulse-labeled with [^{3}H]thymidine after 24 h luminal oxalate or COM crystals. The total number of cells left on the filters after incubation with oxalate was quantified by counting the cells in a hemocytometer.

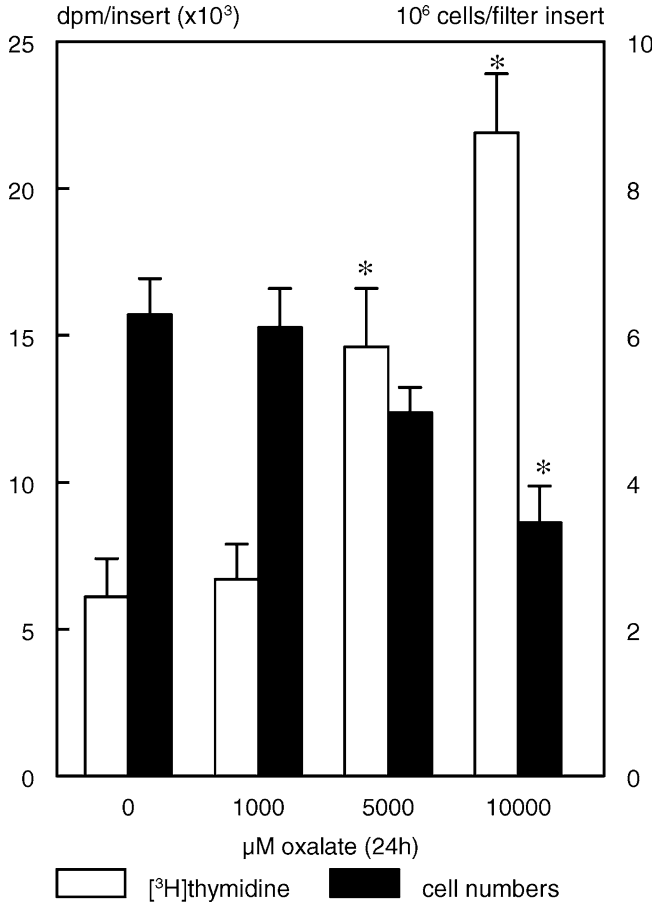

Fig. 5 Confluent MDCK-II monolayers were cultured on porous supports in a two-compartment culture system and incubated with 0, 1,000, 5,000 or 10,000 μM oxalate. Oxalate was added to the apical compartment in a calcium-free buffer, while the basal compartment received DMEM 10% FCS. After a 24 h incubation period the cells were pulse-labeled with [^{3}H]thymidine and in a parallel series of transwells released with trypsin and counted in a hemocytometer. There is an inverse relationship between DNA synthesis and total cell numbers. Means ± SD of a representative experiment are shown. Statistical analysis was performed using ANOVA ($P < 0.05$, $n = 3$). An *asterisk* indicates significantly different compared to untreated controls

Oxalate

Compared with untreated controls, there was no effect of high oxalate (1,000 μM) on [^{3}H]thymidine incorporation or total cell numbers in either cell line. The incorporation of [^{3}H]thymidine was significantly increased in MDCK-II cells exposed to ≥5,000 μM oxalate. This increase in DNA synthesis was accompanied by a decrease in total cell numbers (Fig. 4). Whereas total cell numbers were also lower in MDCK-I cells treated with extremely high oxalate, another day was required to measure elevated levels of [^{3}H]thymidine incorporation (not shown).

Crystals

Although the incorporation of [^{3}H]thymidine was not increased in MDCK-II cells incubated for 24 h with COM (in dpm/insert; 5,130 ± 270 in controls, versus 5,630 ± 210 in COM), DNA synthesis was significantly higher 1 day later (3,950 ± 230 in controls, versus 4,970 ± 550 in COM). COM did not affect DNA synthesis in MDCK-I cells (not shown).

Apoptosis and necrosis

To determine whether oxalate and/or crystals induce physiological or pathological cell death, we studied the oxalate or crystal-induced appearance of phosphatidylserine (annexin V; apoptosis) at the outer leaflet of the lipid bilayer, DNA fragmentation (Hoechst; apoptosis) and PI influx (necrosis).

Oxalate

Hoechst staining showed that high (1,000 μM) or extremely high (≥5,000 μM) oxalate did not induce DNA fragmentation in any cell type (Fig. 5). Oxalate also did not increase the binding of annexin V to the cell surface (Fig. 5). The fact that our cells were capable of undergoing apoptosis was demonstrated using antimycin A (Fig. 5). Irrespective of the cell line used, only extremely high oxalate concentrations were able to permeabilize the plasma membrane (≥5,000 μM oxalate) (Fig. 5).

COM

The internalization of COM by proximal tubular cells, at least in some of the cells, resulted in necrotic cell death (Fig. 6). Hoechst staining revealed that the DNA was not fragmented and that crystals also did not lead to higher levels of annexin V binding, indicating that crystals did not trigger programmed cell death (Fig. 6). Crystals did not induce any form of cell death in collecting tubule cells (not shown).

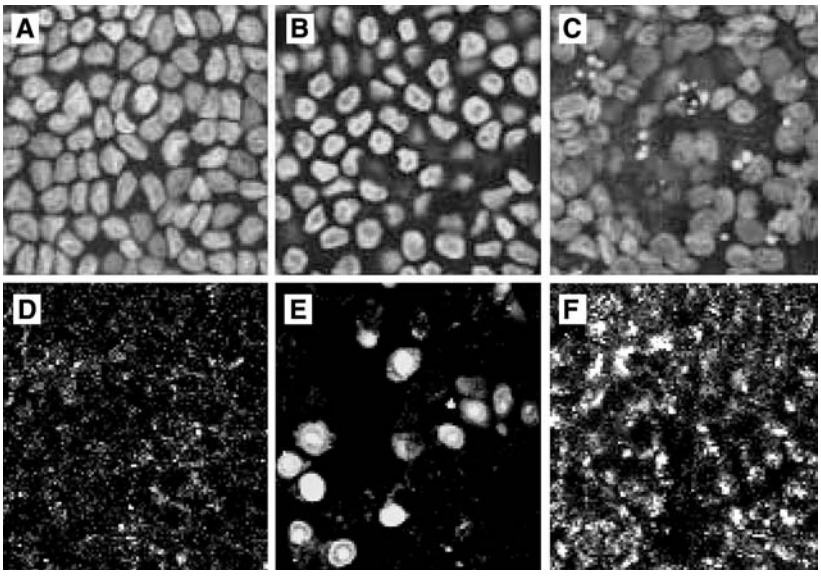

Fig. 6 MDCK cells exposed for 24 h to 0 (**a** and **d**) or ≥5,000 μM oxalate (**b** and **e**), or to 1 μM antimycine A (AA) to induce apoptosis (**c** and **f**). To detect physiological (apoptotic) or pathological (necrotic) cell death, MDCK-II cells were stained with Hoechst (DNA fragmentation) (**a**, **b**, **c**), or propidium iodide (PI influx) and annexin V (phosphatidylserine; PS exposure) (**d**, **e**, **f**). Although there is a reduction in the total number of cells treated with high oxalate there is no DNA fragmentation (**b**) in contrast to cells treated with AA (**c**). Untreated controls contain very few annexin V positive cells and there is hardly any PI influx (**d**), treatment with high oxalate leads to PI influx but not to annexin V binding (**e**). AA triggers the cell surface exposure of PS but does not influence the influx of PI (**f**)

Discussion

This study was performed to test the concept that oxalate and/or calcium oxalate crystals provide stress conditions that are conducive to renal stone formation. The results show that luminal oxalate is not very toxic to renal tubular cells in culture. Inflammation-mediated necrotic cell death is observed only after exposing the cells for a long period of time (24 h) to extremely high oxalate concentrations (≥5,000 μM). The release of necrotic cells was accompanied by an increased incorporation of thymidine in the nucleic acids of the remaining cells, indicative for regeneration. Our study also showed that calcium oxalate crystals rapidly bind to and are taken up by renal proximal tubular cells to induce inflammation-mediated necrosis. The response to crystals was less marked and without cell death in renal collecting tubule cells. In contrast to our predictions, there was no clear difference in oxalate sensitivity between cells on plastic and permeable growth substrates, between proximal and collecting tubule cells, or between subconfluent and confluent cultures. Oxalate was more toxic from the basal membrane, but this difference was also not very impressive (not shown). PGE_2 was secreted predominantly into the apical fluid compartment (not shown). Since we did not exactly copy the experimental design used by others, it should be considered that our cells were cultured on permeable growth substrates, that they received calcium and serum from the basolateral, but not from the apical plasma membrane and that the exclusive addition of oxalate to the apical compartment resulted in some apical-to-basal oxalate leakage.

We and others adhere to the concept that renal stone disease should be considered an inflammation-mediated disorder [10–12]. Stone formation requires crystal formation followed by crystal retention in the kidney. In our view, crystal formation and retention are two entirely different entities. Formation depends on physicochemical mechanisms, such as supersaturation and nucleation. Retention depends on cell biological mechanisms, such as alterations in the architecture of the cell surface leading to crystal attachment. Previously, we demonstrated that under certain circumstances non-adherent renal tubular cells can be transformed into crystal binding cells. The crystal-binding phenotype is characterized by its luminal expression of CD44, osteopontin and hyaluronan [13, 14]. Renal calcification results from an unfortunate coincidence of circumstances during which both crystal formation and retention are promoted. Thus, there are individuals with heavy crystalluria who do not form stones because there is no crystal retention, and there are individuals with renal tubular cells which are susceptible to crystal binding and who do not form stones because their urine is not concentrated enough.

The effects of oxalate or calcium oxalate crystals on renal tubular cells described in the literature are remarkably similar. Both oxalate ions and calcium oxalate crystals trigger DNA synthesis and proliferation [6, 15–17], free radical production [7, 18–21], cell damage [5, 22, 23], oxidative stress [20, 24, 25], p38 mitogen-activated protein kinase (MAPK) [26, 27], MCP-1 expression [28] and apoptotic [29, 30] or necrotic cell death [26, 30, 31]. Oxalate and crystal toxicity studies, however, have

their specific pitfalls. Oxalate toxicity has been studied predominantly with renal tubular cells grown on tissue culture plastic. The disadvantage of this method is that oxalate rapidly forms crystals with calcium in the growth medium [32] and crystals may contribute to the effects attributed to oxalate. Crystal-cell interaction studies have been performed with various cell types including, wild-type MDCK [5, 23, 24, 30, 33], LLC-PK1 [20, 22, 24, 27, 30], OK-1 [34], BSC-1 [35–38] and NRK52E cells [28, 29, 39]. Wild-type MDCK, however, is composed of at least two strains, MDCK-I and MDCK-II with entirely different properties [40–43]. Furthermore, LLC-PK1 and OK-1 are representative for the proximal tubule and the origin of BSC-1 and NRK52E is unknown. Thus, crystal toxicity was studied in various cell types, but not in those derived from segments where crystals frequently occur (i.e. the late nephron).

In the present study we investigated the possible damaging effect of oxalate anions and calcium oxalate crystals, independently, on cell types resembling those of the renal proximal and collecting tubules. The results show that free oxalate is cytotoxic at about ten times its maximum physiological concentration and that cells that frequently encounter crystals (collecting tubule) are practically invulnerable to them. Based on these results, it is unlikely that oxalate ions or calcium oxalate crystals can serve as offending stimuli in the kidney.

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