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OXALATE-INDUCED DAMAGE TO RENAL TUBULAR CELLS

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Abstract

Our own studies and those of others have shown that the incidence of calcium oxalate stones and plaques is markedly increased by nephrotoxins. The possible role of oxalate as a nephrotoxin has not been fully appreciated. However, recent studies in experimental animals and in cultured cells support this possibility. The results of these studies led us to hypothesize that hyperoxaluria promotes stone formation in several ways: by providing a substrate for the formation of the most common form of renal stones, calcium oxalate stones, and by inducing damage to renal epithelial cells. Damaged cells in turn would produce an environment favorable for crystal retention and provide membranous debris that promotes crystal nucleation, aggregation and adherence. The present report summarizes evidence for oxalate nephrotoxicity and discusses the potential importance of oxalate toxicity in the pathogenesis of stone disease.

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Introduction

The relative importance of hyperoxaluria in stone disease has received increased attention in the past few years (Hodgkinson and Wilkinson, 1974; Butz and Kohlbecker, 1979; Tiselius, 1980; Lemann, 1993). Indeed, current evidence indicates that the probability of stone formation and the severity of stone disease are directly proportional to urinary oxalate levels, even within the normal range (Hodgkinson and Wilkinson, 1974; Butz and Kohlbecker, 1979; Lemann, 1993). The precise mechanism by which hyperoxaluria promotes stone formation is not entirely clear. Part of the explanation lies in effects on urinary calcium oxalate saturation since changes in oxalate are reportedly 15 times more potent than equimolar changes in calcium for calcium oxalate saturation (Robertson, 1976; Finlayson et al., 1984). However, recent evidence suggests that oxalate may contribute to stone formation in an additional way: by producing damage to renal tubular cells.

Evidence for oxalate nephrotoxicity was first obtained in experimental models of stone disease. Studies by Khan and Hackett using rats treated with hydroxyproline or ethylene glycol (Dykstra and Hackett, 1979; Khan et al., 1979, 1982; Khan and Hackett, 1993) as well as our own studies using rats fed diets enriched with ammonium oxalate (Menon et al., 1989; Kumar et al., 1991) demonstrated that hyperoxaluria is accompanied by enzymuria and membranuria, a finding consistent with damage to renal tubular cells (Khan et al., 1989, 1992; Khan and Hackett, 1993). Moreover, these changes were often observed even in the absence of overt crystalluria, suggesting that the oxalate-induced damage was not simply due to mechanical injury produced by crystals (Khan et al., 1992; Khan and Hackett, 1993).

Much of the injury produced by hyperoxaluria apparently occurred in the renal epithelial cells as evidenced by the appearance in the urine of tubular cell enzymes (alkaline phosphatase, a brush border enzyme, and N-acetyl-β-glucosidase, a lysosomal enzyme). Similar findings (i.e., enzymuria) have also been observed in patients with idiopathic calcium oxalate stones, although

the results in patients are harder to interpret (i.e., damage could be due to mechanical obstruction/injury; Baggio *et al.*, 1983).

These findings prompted us to assess the effects of oxalate on renal cellular function in more detail; and our recent studies using LLC-PK1 cells, a line of proximal tubular cells from porcine kidneys, provided further evidence for oxalate-induced damage to renal cells (Scheid *et al.*, 1994; Menon *et al.*, 1993). As reported below, these studies demonstrated deleterious effects of oxalate on cellular morphology, on the ability of the cells to exclude vital dyes and on cell survival after prolonged exposure to elevated levels of oxalate ($\geq$ 400 μM free oxalate). These studies also provided insights as to the possible mechanisms underlying oxalate nephrotoxicity.

Materials and Methods

Cell culture

LLC-PK1 cells (CRL 1392, American Type Culture Collection, passage 205-240) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, glucose and antibiotics at 37°C, under 5% CO_2. Experiments were conducted in either DMEM (buffered with either bicarbonate or HEPES) supplemented with 0.25% serum, or mammalian Kreb's Ringer (MKR) buffer containing: NaCl: 122 mM; KCl: 3 mM; $MgCl_2$: 2.25 mM; HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid): 24 mM; $NaHCO_3$: 15 mM; $CaCl_2$: 1.8 mM; dextrose: 10 mM; pH: 7.2. DMEM, penicillin and streptomycin were obtained from GIBCO (Grand Island, NY), fetal bovine serum from Hyclone Laboratories (Logan, UT). All chemicals were of the highest grade available.

Morphological studies

LLC-PK1 cells were grown to confluence in 6 or 12 well plates and treated for varying periods with DMEM-HEPES (pH 7.4) containing oxalate (0-2 mM total, 0-750 μM free, estimates of free oxalate in various buffers calculated using the EQUIL program, see, Werness *et al.*, 1985). Cells were then fixed (5 minute exposure to 4% paraformaldehyde in 0.25 M phosphate buffered saline, pH 7.4) and stained with hematoxylin and eosin (H&E). Fields of cells were selected at random by drawing a small circle in the center of each segment of the chamber. The culture plate was then transferred to the stage of a microscope and the cells within the circle were photographed at 200 or 400 X.

Cell viability assay - vital dyes

LLC-PK1 cells were grown to confluence in 6 or 12 well plates, the culture medium was replaced with DMEM containing varying concentrations of oxalate for 1 or 4 hours. At the end of this period, cells were incubated for 5 minutes with phosphate buffered saline containing 0.05% trypan blue. Cells were then examined under a light microscope and the relative abundance of dead (stained) cells was assessed for each condition. Other experiments utilized ethidium homodimer-1, a fluorescent vital dye that is excluded from intact cells, for quantitating the number of dead cells. For such studies, LLC-PK1 cells were grown to 50-75% confluence in 48 or 96 well plates. Cells were treated for 4 hours at 37°C in MKR with varying oxalate concentrations (0-1 mM total, 0-350 μM free) and stained with 50 μl of a mixture containing 4 μM ethidium homodimer-1 (which stains dead cells; Haugland, 1992) and 15 nM Hoechst 33342 (which stains all cells; Haugland, 1992). Staining involved addition of 50 μl of dye solution to each well and incubation at 37°C for 30 minutes. A minimum of 6 wells with two randomly selected fields in each well was then examined using a fluorescence microscope (Nikon). Each field of cells was photographed twice, once with a filter set for ethidium homodimer-1 (excitation wavelength, λ: 525 nm; emission λ: 600 nm) and once with a filter set to detect Hoechst 33342 (excitation λ: 340 nm; emission λ: 480 nm). The number of dead cells per field was counted and the effects of various treatments were analyzed for statistical significance using a Fisher's Exact test.

Monitoring free radical production-Spectrofluorimetry

To assess the effects of oxalate on the production/availability of free radicals, LLC-PK1 cells were loaded with dihydrorhodamine 123 (DHR), a non-fluorescent compound that is converted to a fluorescent product, rhodamine 123, by reactive oxygen molecules (Rothe *et al.*, 1988; Emmendorffer *et al.*, 1990; Henderson and Chappell, 1993). For such studies, confluent cultures of LLC-PK1 cells on rectangular 12 x 25 mm coverslips were removed from their culture medium, washed once with MKR and transferred to a standard cuvette containing MKR with 1-10 μM DHR. After 2 minutes exposure to dye-containing solution, the coverslip was removed, the cuvette was rinsed 3 X with MKR, and refilled with MKR. The coverslip was then placed diagonally across the cuvette, and the fluorescent signal at 540 nm was detected at right angles to the excitation source (λ = 510 nm). Baseline fluorescence was recorded for a minimum of 10 minutes, then the effects of various additives (30 μl additions) were monitored for an additional 10 minutes. Further additions were made at 10 minute intervals. The final addition was always 30 μl of 0.8 M hydrogen peroxide (a positive control to demonstrate that cells retained sufficient dye). All experiments were conducted at 22 $\pm$ 1°C, fluorescence in-

tensity was recorded each second and the rate of change in fluorescence was analyzed by linear regression analysis of the last 5 minutes of the 10 min interval. The results reflect an average of at least 8 experiments for each experimental condition. Because there was considerable variation among experiments even under basal conditions, each coverslip served as its own control, and statistical significance of the results was tested using a standard paired t-test with a confidence level of 0.025.

Results

Morphological alterations

Morphological studies provided the initial evidence for oxalate toxicity in renal epithelial cells. Exposure of LLC-PK1 cells to oxalate led to changes in microscopic appearance of the cells that were consistent with cellular injury (Fig. 1). Exposure to moderate levels of oxalate (400 μM total oxalate, 140 μM free oxalate) led to an increase in the incidence of cells exhibiting cytoplasmic vacuolization (Fig. 1B) while exposure to higher levels of oxalate (1 mM total, 350 μM free) increased the incidence of cells exhibiting pyknotic nuclei (Fig. 1C). By comparison, the untreated cells exhibited a typical epithelial morphology (Fig. 1A) as reported previously (Hull et al., 1976; Cantiello et al., 1986). The effects of oxalate were both time and concentration dependent with prolonged exposure (60 minutes) to high concentrations (350 μM free oxalate) producing the most severe damage to the renal epithelial cells.

Membrane permeability changes

These changes in morphology were accompanied by alterations in membrane permeability as evidenced by increased uptake of vital dyes. In the representative experiment in Figure 2, subconfluent cultures of LLC-PK1 cells were incubated in the presence of varying concentrations of oxalate (0, 140 or 350 μM free oxalate) for 1 hour. Cells were then stained with trypan blue. Note that oxalate exposure markedly increased the number of dead cells (darkly stained) in this experiment (compare Figs. 2B and 2C with Fig. 2A). Indeed pooled data from several such experiments indicated that a 4 hour exposure to 350 μM free oxalate increased the number of dead cells 3-4 fold, from 6.5 $\pm$ 2.3 dead cells/field in untreated controls to 21.5 $\pm$ 3.8 dead cells/field in oxalate-treated cultures (data are means $\pm$ standard error of mean, SEM, from 12 wells with duplicate measurements for each well and include results from at least 3 separate cell platings; p < 0.05.).

Decline in cell numbers

To determine whether or not the observed changes in membrane permeability were indeed irreversible, we examined cell density in cultures after prolonged exposure to oxalate. Subconfluent cultures of LLC-PK1 cells were grown for 3 days on multiwell plates containing medium supplemented with 0.25% or 10% serum plus varying amounts of oxalate (0, 350 μM, 750 μM or 1.6 mM free oxalate; 0, 1, 2 or 4 mM total oxalate). Cells were then fixed and stained with crystal violet, and the dye content of each well was determined spectrophotometrically (Barer et al., 1986; Koul et al., 1994). Separate studies confirmed that dye content was proportional to cell density, thus, oxalate-induced cell loss was detected as a decline in the staining of treated cultures relative to controls. As can be seen in Figure 3, prolonged exposure to high levels of oxalate produced a concentration-dependent decline in cell numbers, with significant losses seen at concentrations $\geq$ 350 μM free oxalate (p < 0.05 in all cases).

Mechanism(s) of oxalate-induced injury

The exact mechanisms involved in oxalate toxicity have not been determined. Oxalate may exert direct effects on cellular metabolism. Alternatively, or in addition, oxalate actions may be secondary to the formation of crystals. A number of studies have suggested that calcium oxalate crystals can induce cellular injury. Studies on red cells and on mononuclear cells (Elferink and Riemersma, 1980; Wiessner et al., 1986) indicated that calcium oxalate crystals can induce hemolysis (although the effects of crystals may have been secondary to internalization and dissolution of crystals since cytolysis was blocked by agents that inhibit endocytosis, Elferink and Riemersma, 1980). Crystal-induced injury has also been reported in cultured renal tubular cells. Exposure of Madin Darby canine kidney (MDCK) cells to urate led to the release of lysosomal enzymes and prostaglandin E2 and, to a lesser extent, cytosolic enzymes (Emmerson et al., 1990; Hackett et al., 1994).

Studies in experimental animals have also suggested that crystal formation may induce tubular injury. Experimental treatments that induce intratubular formation of calcium oxalate crystals also induce the focal loss of brush border from proximal tubular cells and the appearance of proximal tubular enzymes and membranes in urine (Khan et al., 1982; 1989; 1992; Menon et al., 1989; Khan and Hackett, 1993). Whether or not crystals play a role in the toxic effects of oxalate on LLC-PK1 cells remains unclear. We did observe calcium oxalate crystals in oxalate-treated cultures. However, there was no consistent link between crystal adherence and overt damage; some dead cells (i.e., trypan blue-stained cells) had adherent crystals, most did not. Rather our studies on LLC-PK1 cells suggested that oxalate toxicity might involve alterations in cellular metabolism.
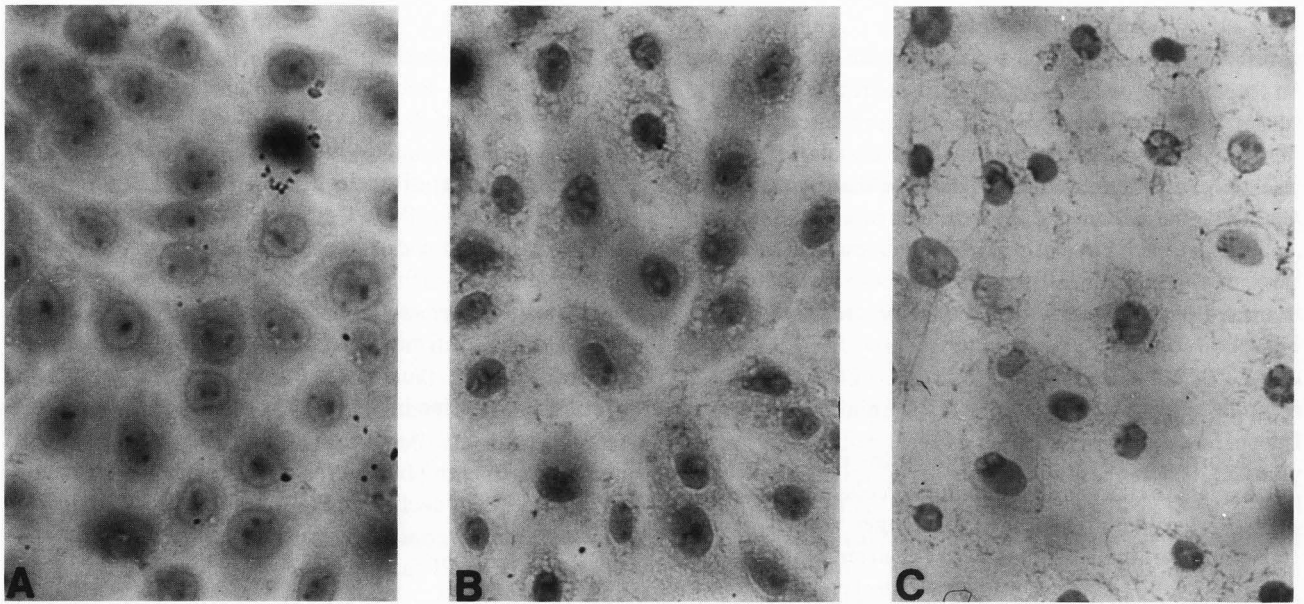
Figure 1. Effects of oxalate on the morphology of LLC-PK1 cells, concentration dependent effects. Cells were exposed to 0 (**A**), 140 (**B**), or 350 (**C**) μM free oxalate for 60 minutes. Cells were then fixed, stained with H&E and examined under a light microscope. Micrographs illustrate progressive alterations in morphology in cells exposed to increasing concentrations of oxalate, with cytoplasmic vacuolization and nuclear pyknosis becoming prominent at 140 μM and 350 μM, respectively. Reprinted from Scheid *et al.* J Urol (1995), with permission.
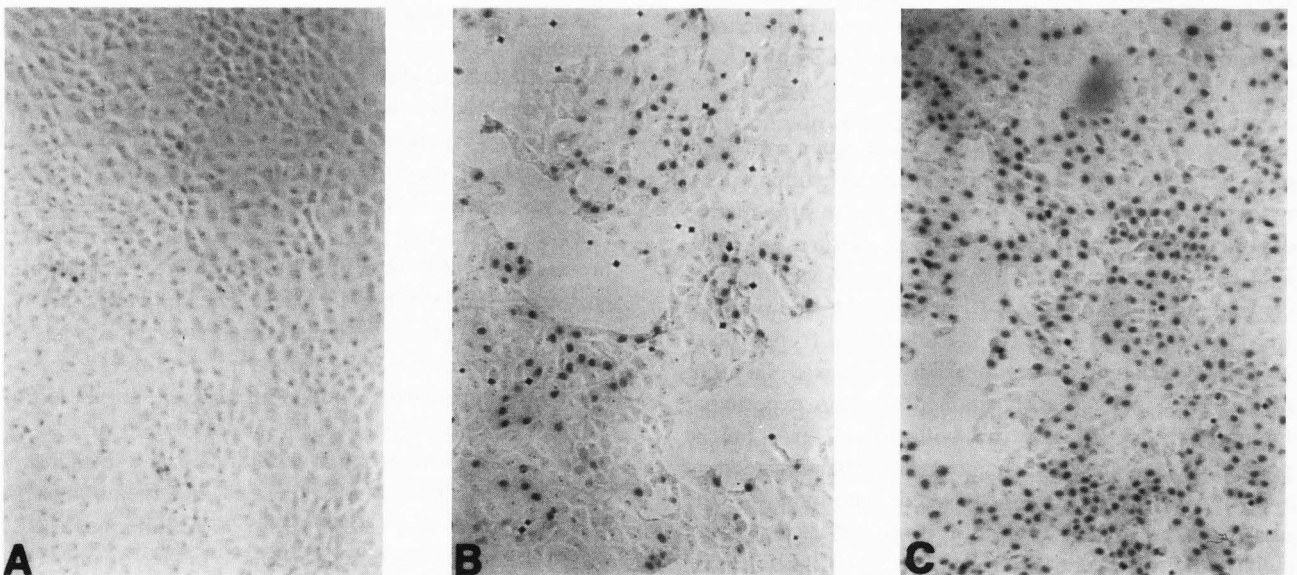

Figure 2 (bottom). Oxalate-induced increase in membrane permeability assessed using trypan blue. In these representative figures, cells were exposed to 0 (**A**), 140 (**B**), or 350 (**C**) μM free oxalate for 60 minutes. Cells were then stained with trypan blue and examined microscopically. Dead/damaged cells were permeant to trypan blue and appear black in these images. Note that oxalate treatment produced a concentration-dependent increase in the abundance of stained cells (compare Figs. 2A and 2B with the control in Fig. 2C). Reprinted from Scheid *et al.* J Urol (1995), with permission.
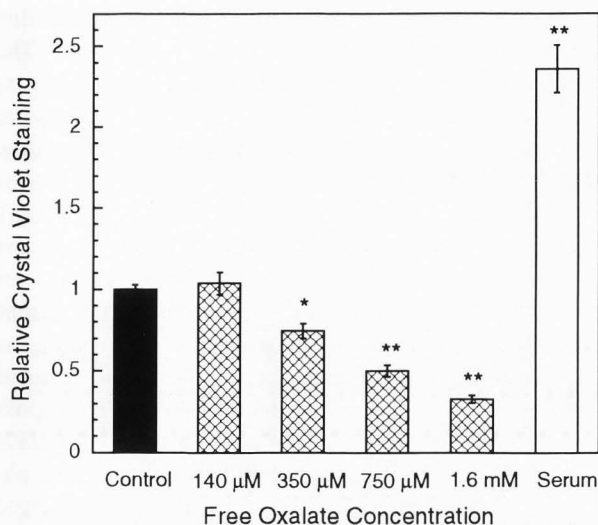
Figure 3. Oxalate-induced changes in cell number. LLC-PK1 cells were serum-depleted (0.25% serum overnight) and then exposed to culture medium containing 0.25% or 10% serum plus varying amounts of oxalate (140 μM to 1.6 mM free oxalate, 400 μM to 4 mM total oxalate) for 72 hours. The effects on cell density were assessed by measuring the extent of crystal violet uptake. Note that exposure to oxalate concentrations above 350 μM (free) led to a significant dose-dependent decline in cell numbers. Data reflect mean changes from control for 55-150 separate determinations performed in 13 different experiments.

Oxalate-induced increases in free radical production

Oxalate esters have been shown to induce free radical formation *in vitro* (Sherman *et al.*, 1978), and a number of nephrotoxic agents induce renal injury via processes involving free radicals (Lash and Anders, 1986; Walker and Shah, 1988; Chen *et al.*, 1990). Thus, we considered the possibility that oxalate toxicity might involve increased production/availability of free radicals. To assess this possibility, confluent monolayers of LLC-PK1 cells were loaded with dihydrorhodamine 123 (DHR), a non-fluorescent dye that is converted to a fluorescent product, rhodamine 123, by free radicals (Rothe *et al.*, 1988; Emmendorffer *et al.*, 1990; Henderson and Chappell, 1993). The rate of free radical production was estimated from the rate of increase in dye fluorescence (Rothe *et al.*, 1988; Emmendorffer *et al.*, 1990; Henderson and Chappell, 1993); and, as can be seen in Figure 4A, exposure to oxalate produced a concentration-dependent increase in the rate of free radical production, with 350 μM oxalate increasing the
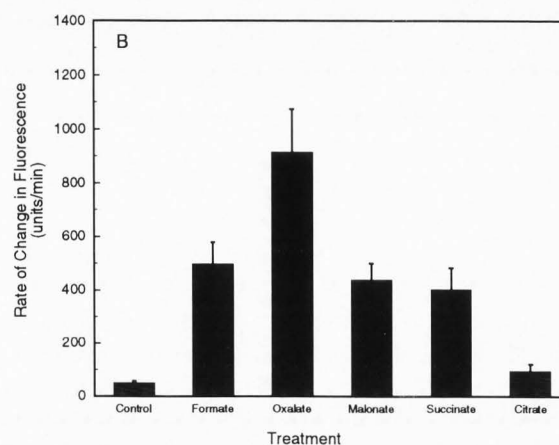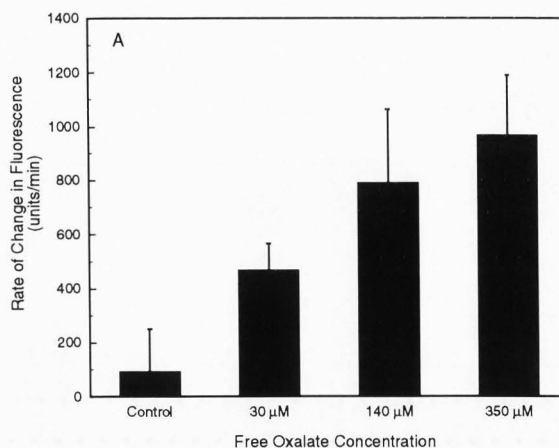
Figure 4. Effect of oxalate on free radical production. LLC-PK1 cells were loaded with DHR and fluorescence was monitored using a SPEX CM1 spectrofluorimeter. **Figure 4A.** Concentration dependent changes in DHR fluorescence. LLC-PK1 cells were exposed to 0, 30, 140 or 350 μM free oxalate for 10 minutes and the rate of change in DHR fluorescence was determined from the slopes of the fluorescence traces. Increasing oxalate levels from 0 to 350 μM led to a progressive increase in the rate of DHR cleavage with significant changes in rate observed at concentrations of 140 or 350 μM free oxalate. Data are means $\pm$ SEM from at least 6 separate experiments. **Figure 4B.** Effect of structurally related mono- and dicarboxylic acids on DHR fluorescence. Note that oxalate was more than twice as potent as the other compounds tested despite the fact that free concentrations of oxalate (350 μM) was lower than that of the other compounds tested (all added at 1 mM). Data are means $\pm$ SEM from at least 4 experiments for each analogue; p < 0.05 for all compounds except citrate.
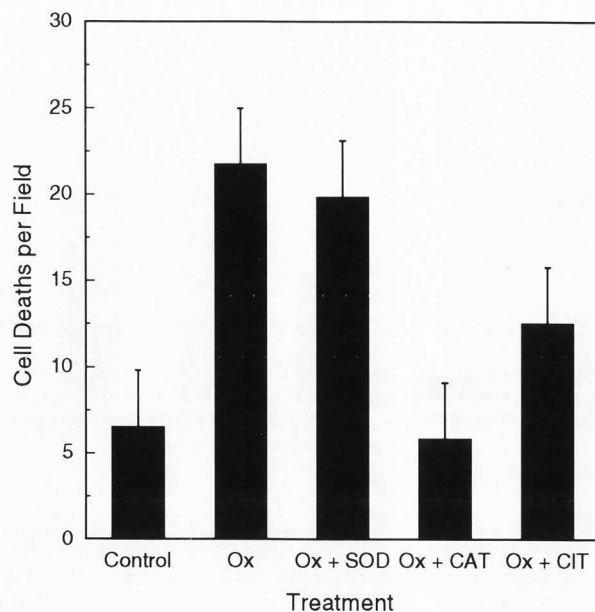
Figure 5. Attenuation of oxalate toxicity by free radical scavengers. LLC-PK1 cells were exposed to oxalate + free radical scavengers for 4 hours and then stained with a combination of Hoechst 33342 and ethidium homodimer-1 (see text). Note that exposure to oxalate (350 μM) increased the number of dead cells per field ~3.5 fold. Addition of catalase (310 U/ml) in conjunction with oxalate prevented this response (p < 0.05 versus oxalate alone). Addition of superoxide dismutase (80 U/ml) along with oxalate did not attenuate oxalate toxicity. Data reflect means ± SEM from at least 6-9 separate cell cultures with duplicate measurements in each culture. For statistical analysis, data from each experiment were normalized relative to control prior to the analysis of variance and Fisher test.

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rate of dye cleavage 8 fold. Other mono- and dicarboxylates were less effective (Fig. 4B). Thus, the effects of oxalate appeared to be selective.

Link between free radical production and toxicity

To determine whether or not the oxalate-induced change in free radical production plays a role in oxalate-induced damage to renal tubular cells, we examined the effects of free radical scavengers on oxalate toxicity. Specifically, we investigated whether or not addition of agents such as catalase (which degrades peroxide) or superoxide dismutase (which converts superoxide to peroxide) would prevent the oxalate-induced increase in membrane permeability that was detected using vital dyes. Such studies demonstrated that simultaneous addition of catalase but not superoxide dismutase protected cells from oxalate toxicity (Fig. 5). These findings support the possibility that oxalate toxicity may be secondary to

an oxalate-induced increase in the production/availability of peroxide or a peroxyl radical in LLC-PK1 cells. The manner by which oxalate might increase cellular peroxide is as yet unknown; however, recent *in vitro* studies demonstrated that oxalate inhibits horseradish peroxidase (data not shown).

Discussion

These data provide clear evidence for oxalate nephrotoxicity. They also provide a possible explanation for the significant role of hyperoxaluria in stone disease. Tubular injury has often been mentioned as a predisposing factor for urolithiasis. The paper by Randall (1937) on calcium oxalate plaques noted that regions of the papilla underlying plaques showed evidence of damage, whereas, adjacent areas did not. Subsequent structural analysis of such regions have revealed that plaques are attached to injured epithelium and suggested that the basement membrane and/or the basolateral surface of the epithelial cells were involved in this process (Khan, 1991). Studies carried out on experimental animals also showed a correlation between injury and enhanced crystal binding. Damage to urothelial cells produced by instilling 0.1 N HCl or 5% Triton X-100 into the rat urinary bladder produced a marked increase in the binding of calcium oxalate crystals (Gill *et al.*, 1979; 1980; Khan *et al.*, 1984), with the extent of binding correlating with the extent of damage to the urothelium. Similarly, damage to renal epithelial cells produced by gentamicin led to an increased incidence of plaques on the renal papilla and an increased incidence of crystals within the kidney (Kumar *et al.*, 1991).

Studies *in vivo* have also been supported by studies *in vitro*. Adherence of crystals to monolayer cultures of renal papillary cells was increased markedly by conditions that damaged the epithelium, i.e., binding occurred to regions of the monolayer in which tight junctions were disrupted exposing the basolateral surfaces of the cells and/or the basement membrane (Wiessner *et al.*, 1987; Riese *et al.*, 1988; 1992; Mandel and Riese, 1991). Thus, oxalate-induced damage to renal tubular cells could promote stone formation by producing an environment favorable for crystal retention and growth.

Oxalate-induced damage to renal tubular cells could also promote stone formation by providing cellular debris for crystal formation. Calcium oxalate stones contain an organic core consisting of a mixture of proteins and phospholipids comparable to that in biological membranes (Boyce, 1968; El-Sayed and Coslett, 1977; Khan and Hackett, 1987); and the addition of cellular membranes to artificial urine promotes crystallization, aggregation and heterogeneous nucleation of crystals (Khan *et al.*, 1988, Hackett *et al.*, 1990). Moreover, studies in

experimental animals suggest a link between membranuria and an increased incidence of crystals in urine. For example, in rats treated with ethylene glycol, crystals of calcium oxalate were commonly seen associated with membranous debris within tubular lumina (Dykstra and Hackett, 1979; Khan *et al.*, 1982). In addition, in rats treated with a combination of ethylene glycol plus gentamicin (which itself damages proximal tubular cells, see, Hori *et al.*, 1984), crystalluria was observed at doses of ethylene glycol that produced only modest increases in the relative supersaturation of the urine, suggesting that factors within the urine (presumably the presence of membranous debris) were promoting heterogenous nucleation of crystals (Khan *et al.*, 1990). Similarly, treatment of rats with gentamicin markedly increased the sensitivity of the animals to dietary oxalate loading. A combination of gentamicin plus 3% oxalate was at least two fold more potent at inducing plaque formation than dietary oxalate supplementation or gentamicin treatment alone, presumably as a consequence of proximal tubular damage (Kumar *et al.*, 1991). These findings support the notion that damage to tubular cells promotes crystal formation and retention by providing cellular debris for nucleation, aggregation and adherence of crystals to cells (Khan *et al.*, 1982).

The significance of these findings for normal kidney function is not entirely clear. Toxic effects of oxalate were only observed at free oxalate concentrations ≥ 350 μM, somewhat higher than might be expected to occur within the proximal tubules. Oxalate levels in normal individuals average only 5-10 μM in the glomerular filtrate (Wolther and Hayer, 1982) rising 10 fold by the end of the proximal tubule as fluid is reabsorbed. Levels of oxalate in this region may be increased further by secretion of oxalate (Knight *et al.*, 1981; Senejkian and Weinman, 1982), but the final concentration of oxalate that is achieved has not been determined [available oxalate assays are too insensitive for use on micropuncture samples; hence estimates of regional oxalate levels have been extrapolated from data on other metabolites or derived from measurements on lyophilized samples (Hautmann, 1982)]. Thus, oxalate may not approach toxic levels within the proximal tubule except in individuals with hyperoxaluria.

To summarize, the present studies demonstrated that exposure to high levels of oxalate produce damage to renal epithelial cells as evidenced by morphological alterations, increased uptake of vital dyes and a decline in cell numbers. Other studies suggested that the toxic effects of oxalate might be due to increased free radical production/availability in the renal epithelial cells. These findings support earlier studies *in vivo* which suggested that hyperoxaluria induces tubular injury and pro-

vide a possible explanation for the tight association between hyperoxaluria and stone disease. Further study will be required to confirm these observations in other types of renal epithelial cells and in intact kidneys.

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Discussion with Reviewers

R.L. Hackett: Could you comment on the levels of oxalate employed in these studies and how you arrived at your estimates of the "free" oxalate concentrations? Using the 1995 version of Finlayson's EQUIL to calculate ionic oxalate, we estimate that the highest oxalate concentration tested (1.6 mM "free") corresponds to a relative supersaturation (RSS) of 47, while the lowest oxalate concentration corresponds to a RSS of 14. The lower value seems appropriate, however, the higher value represent a quite elevated oxalate level.

Authors: Our estimates of the "free" oxalate concentrations used an earlier version of the EQUIL2 program. We agree that the highest concentrations tested are not likely to occur within the renal cortex (although they might occur within the renal papilla), but we were interested in using a wide range of concentrations to examine the effects of various doses and exposure times. The studies showed that the effects of oxalate are both time- and concentration-dependent, i.e., even moderate doses of oxalate (160-400 μM free, 400 μM to 1 mM total) can elicit toxicity with prolonged exposure. Thus, individuals with hyperoxaluria and/or renal dialysis patients with hyperoxalemia might be at risk for oxalate toxicity.

R.L. Hackett: Could you comment on the possible effects of other ions and/or proteins in the injury process?

Authors: In the studies depicted in Figure 4, we showed that oxalate is more than twice as effective as structurally related mono- and dicarboxylates at increasing the rate of free radical production in LLC-PK1 cells. Moreover, oxalate was the only compound tested that induced cell death. We have not looked extensively at the effects of other ions or proteins, although we found that citrate provided a partial protection against oxalate-induced cell death whereas 10% serum provided full protection.

R.L. Hackett: In our recent studies (Hackett *et al.*, 1994), we reported that oxalate crystals are toxic for MDCK cells and demonstrated that the addition of oxalate in solution enhanced this effect. Meanwhile, Lieske *et al.* (1992) found that crystals were not toxic for BSC-1 cells and Verkoelen *et al.* (1995) found no evidence of toxicity after treatment of LLC-PK1 cells with CaOx saturated media containing crystals. Could you comment on these discrepancies with respect to your own data. In addition, could you explain your contention that the toxic effects appear to be mediated by the oxalate in solution since it seems likely that the oxalate concentrations that you used might precipitate calcium oxalate crystals.

Authors: There is no simple explanation for the observed differences in the studies that you cited except to state that cell lines can differ markedly. In addition, individual cell lines can be quite capricious and large variations are often observed **even in the same laboratory** depending on the passage level, culture conditions, etc. For example, we find that subconfluent (growing) cultures of LLC-PK1 cells are much more sensitive than confluent (quiescent) cultures to the toxic effects of oxalate. There is also no simple answer as to the relative importance of crystals versus oxalate in solution. Crystals **do** form under the experimental conditions that we employed for these studies and we cannot rule out effects due to crystals. What we found, however, was that dead cells (ie cells stained with vital dyes) were rarely associated with crystals, i.e., there was no apparent correlation between crystal deposition/binding with cell death.

R.L. Hackett: Free radical assays were done at 10 minutes, H&E specimens at 60 minutes, permeability stains at 4 hours, and cell numbers at 3 days. How do you know that the changes are correlated? Cultures may be in different stages with different cell populations. Also, can you explain why you chose these time points for the different assays and indicate how long cells were exposed to free radical scavengers?

Authors: The time points were chosen for practical reasons. Since free radical production rates appeared to remain stable for prolonged periods (hours) after a given treatment, we chose a sampling interval that was sufficiently long to minimize "noise" but not so long as to be burdensome for our technician. Choice of the sampling time for the morphological studies was dictated by the inherent heterogeneity of the cultures and our desire to eliminate observer bias. All cultures have some cells with "aberrant morphology" and oxalate increases the relative abundance of these cells (with higher oxalate concentrations producing these effects more rapidly than lower concentrations). Thus, we chose a time point at which we could reliably detect alterations (at the highest doses) and then examined cells in random regions of the cultures. The studies on membrane permeability have been repeated at shorter intervals (1 and 2 hours) in the past few months. These studies show a similar trend although differences between control and experimental groups is smaller. When radical scavengers were added, they were included for the entire exposure period; indeed, in several studies, we made multiple additions to make sure that the scavengers would be effective for the entire treatment period. Cells appear to withstand a moderate oxalate "insult" for some time but eventually cells succumb (perhaps due to depletion of free radical buffers). Lower levels of oxalate appear to be tolerated even for prolonged periods. Indeed low levels of oxalate promote proliferation of the cells (Koul *et al.*, 1994). That appears to be the reason that we observe biphasic changes in cell numbers after 3 days exposure to oxalate, i.e., oxalate induces both proliferation and death of cells with proliferation (i.e., a net increase in cell numbers) predominating at low doses and death (i.e., a net decline in cell numbers) predominating at the higher doses.

R.L Hackett: In analyzing the crystal violet assay, did the authors take into account the possible binding of exogenous serum binding to crystal violet? What is the viability of the cells that are left? In our experience, oxalate-induced changes in cell viability are focal. Did your live-dead assay which scores random fields of cells take this into account?

Authors: Cultures were washed extensively to remove trace serum, and control studies (using cells plated at different densities) were run to make sure that dye intensity was proportional to cell number. We do not examine the relative abundance of live versus dead cells after 3 days exposure to oxalate. Since crystal violet stains all cells (live and dead), we obtained an estimate of the number of cells/dish that are still attached after treatment. We also found that oxalate-induced changes in viability tended to be focal and our live/dead assay was designed to take this into account, i.e., cells were examined at low magnification (20X) to increase the number of cells/field and fields were selected at random.

N.S. Mandel: How would you correlate your observations on oxalate toxicity in LLC-PK1 cells with Lieske *et al.* (1992) observations in BSC-1 cells that calcium oxalate monohydrate (COM) endocytosis induces cell proliferation but not necessarily cell toxicity.

Authors: We can only speculate, but we think the different responses to COM crystals versus oxalate in solution may relate to the relative amounts of free radicals formed and/or differences in the sites of free radical production. There is no other evidence for crystal-induced changes in free radical production in renal epithelial cells; however, this phenomenon has been documented in endothelial cells where endocytosis of crystals leads to increased free radical production (Falasca *et al.*, 1993). This appears to be mediated by a plasmalemmal enzyme (an NADPH oxidase) and the free radicals produced may be more restricted than those produced in response to oxalate uptake by carriers.

N.S. Mandel: Do you think that the rapid removal of serum in your experimental conditions might have compromised the health of the cells in such a way as to exaggerate your oxalate toxicity observations? And, is

the effect of rapid serum removal on cell health concentration dependent?

Authors: The cultures seem more sensitive to the type of buffer than the level of serum in short term (several hours) experiments; i.e., more damage was observed in serum-free MKR than in serum-free DMEM. We have not tried to sort out which ingredients in the latter are most beneficial. For long term experiments, some serum is required (0.25%) but cells can be maintained for long periods in media containing low serum. For example, in studies on cell number there was no decline in controls over 72 hours, although cell numbers did decline in cultures treated with high levels of oxalate. High concentrations of serum **will** protect against oxalate toxicity, and it is not clear whether the serum simply "buffers" the free radicals and/or exerts other effects on cell metabolism. We also have found that confluent cultures are more resistant to oxalate than subconfluent cultures, so there are a number of factors that dictate oxalate sensitivity.

C. Verkoelen: Since the oxalate levels you employed are relatively high and likely to occur only in the collecting duct and in the papilla, could you comment as to why you selected a proximal tubular cell line for these studies?

Authors: We think it likely that "upstream" damage to proximal tubular cells plays an important role in stone formation in that damaged cells can provide cellular debris to serve as heterogeneous nucleators of COM crystals. The doses of oxalate used in these studies **are** somewhat high and we need to look at the effects of longer term exposures to lower levels of oxalate to more closely mimic events *in vivo*. We also plan to look at other cell lines since damage to more distal regions appears to promote crystal adherence (although the mechanisms are likely to be different in the distal regions of the kidney).

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Note added in proof: In recent studies (submitted to Kidney International), using higher concentrations of superoxide dismutase (SOD; 500 U/ml), we found that SOD blocked oxalate-induced increases in free radical production and in cell death. This suggests that oxalate actions may be initiated by increases in superoxide in LLC-PK1 cells.