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Effects of Oxalate on IMCD Cells:

A Line of Mouse Inner Medullary Collecting Duct Cells

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Abstract

Oxalate, a metabolic end product and a major constituent of the majority of renal stones, has been shown to be toxic to renal epithelial cells of cortical origin. However, it is unknown whether inner medullary collecting duct (IMCD) cells that are physiologically exposed to higher concentrations of oxalate also behave in a similar manner. In the present study, we examined the effects of oxalate on IMCD cells. IMCD cells from the mouse were maintained in DMEM/F12 media supplemented with fetal bovine serum and antibiotics. Exposure of IMCD cells to oxalate produced time- and concentration-dependent changes in the light microscopic appearance of the cells. Long-term exposure to oxalate resulted in alterations in cell viability, with net cell loss after exposure to concentrations of 2 mM or greater. The production of free radicals was directly related to the exposure time and the concentration of oxalate. Crystal formation occurred in less than 1 h and cells in proximity to crystals would lose membrane integrity. Compared with IMCD cells, LLC-PK1 cells as well as HK-2 cells showed significant toxicity starting at lower oxalate concentrations (0.4 mM or greater). These results provide the first direct demonstration of toxic effects of oxalate in IMCD cells, a line of renal epithelial cells of the inner medullary collecting duct, and suggest that the cells lining the collecting duct are relatively resistant to oxalate toxicity.

Keywords

oxalate; IMCD cells; toxicity; free radicals

INTRODUCTION

Oxalate is an organic dicarboxylate and is primarily excreted by the kidney. It is a major constituent of a majority of urinary tract stones. Oxalate-induced alterations in renal epithelial cells are the critical event in crystal retention and clinical stone disease. We demonstrated that oxalate is toxic to renal epithelial cells and that exposure of renal epithelial cells to oxalate resulted in selective activation of p38 mitogen-activated protein kinase.^{1–3} Moreover, we observed a net cell loss at high levels of oxalate despite increased DNA synthesis. The mechanics and modification of stone initiation are the subject of intense investigation. Inner medullary collecting duct (IMCD) cells survive in a unique environment within the organism, commonly experiencing otherwise lethal extremes of osmolality, pH, and toxins. These cells also experience higher concentrations of oxalate than cortical epithelial cells. In the present study, we examined the effects of sodium oxalate in a line of mouse IMCD cells and demonstrated a relative resistance to oxalate toxicity compared with renal cells of cortical origin.

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METHODS

Cell Culture

For these studies, IMCD cells were obtained from the American Type Culture Collection (Bethesda, MD). Cells were serially passaged in T-75 flasks, plated at high density on six-well plates, and grown in DMEM supplemented with serum (10%) and antibiotics at 37°C in a 5% CO₂-supplemented environment. For experiments, cells were grown on 12- or 24-well plates or 8-chamber slides at subconfluence or confluence as specified. A stock solution of 50 mM sodium oxalate was made in PBS, filtered, and used within 5 days at concentrations of 0.2, 0.5, 1, 2, 4, or 10 mM.

Assessment of Cell Number Using Crystal Violet

IMCD cells were grown to 70–80% subconfluence. The medium was replaced with an identical serum-free variety after one wash and incubated for another 14 h. Oxalate was added to the wells. At the specified time points, crystal violet stain was added directly to the wells and allowed to stain for at least 20 min. Plates were then rinsed gently and allowed to air dry. The dye was extracted overnight with 2-ethoxyethanol, and optical density at 595 nm (Bio-Rad Benchmark Micro-plate Reader) was used as a relative indicator of cell number.

Hematoxylin and Eosin Staining

Cells on eight-chamber slides were exposed to oxalate for 24 or 48 h. The chambers were washed and fixed with 3.7% formalin in PBS. Cells were then stained with hematoxylin and eosin.

Estimation of Free Radical Production

IMCD cells were grown as described above to near confluence. Cells were then kept in serum-free medium for 14 h. Nitroblue tetrazolium (0.1 mg/mL) was added immediately before oxalate exposure. The oxidative dye reaction was stopped with 70% methanol, and wells were adequately washed with 100% methanol before drying. The intracellular oxidized dye was then extracted with a 1:1.167 mixture of 2 M KOH and DMSO. Samples were transferred to microfuge tubes and centrifuged for 5 min at 14,000 rpm. Absorbance was then measured at 655 nm and indexed for free radical production.

Membrane Integrity Using Vital Dyes

IMCD cells grown on eight-chamber slides were exposed to oxalate for 45 or 105 min before addition of trypan blue stain. The stain was allowed to set for 15 min before aspiration of the stain/medium combination. The chambers were then washed with PBS several times and placed in 3.7% formalin in PBS. Cells were observed microscopically for nuclear uptake of blue dye. Cells that included the dye were counted in five (200×) high-powered fields in each treatment and averaged. After a period of fixation in 3.7% formalin, the cells were stained with eosin.

RESULTS AND DISCUSSION

This mouse IMCD cell line grew more quickly in culture than other renal epithelial cell lines previously used in our laboratory, which include LLC-PK1, MDCK, and HK-2.⁴ Cells would continue to divide for a short period of time after serum starvation, which suggests a certain durability of this cell line and contributed to difficulty in arresting growth and division. An overnight growth from 80% subconfluence to full confluence in serum-free medium was commonly observed.

Oxalate caused a concentration-dependent reduction in cell density by crystal violet staining. Relative loss of absorbance was observed at concentrations of 2 mM or greater, and was in direct relation to the amount of oxalate (data not shown). Lower amounts of oxalate (0.2, 0.5, and 1 mM) did not demonstrate cell loss by crystal violet staining. Photomicrographs taken after hematoxylin and eosin staining (Fig. 1) demonstrated calcium oxalate monohydrate and dihydrate crystal formation in all oxalate-treated wells. The size and number of crystals were in direct proportion to concentration, as was observed for cell loss. At 10 mM sodium oxalate, viable-appearing cells by microscopy were scant. No significant change in cell density was observed at concentrations up to 1 mM by both crystal violet staining and direct microscopic observation. Free radical production was determined using nitroblue tetrazolium. Optical densities measured in each experimental sample were acceptable at 0.05–0.12. The shortcoming of dye precipitation at higher concentrations prevents reliability at increased absorbance. Good reliability was observed in each experimental group.

There was a time- and concentration-dependent increase in reactive oxidative species (ROS) in IMCD cells. The relationship was generally linear over the 4-h period when all of the oxidized dye was included. When accounting for background production of ROS (assumed from control), the highest production of free radicals occurred in the 4 mM-treated group at 60 to 120 min. Generation of ROS was directly related to oxalate concentration. By nature of the protocol, the ROS produced are intracellular, as ROS released extracellularly are washed away. In cells treated with oxalate, there was a dose-dependent increase in trypan blue staining. Calcium oxalate monohydrate and dihydrate crystals formed within 1 h of treatment. Under microscopic view, cells near crystal aggregates had increased dye inclusion. There were sporadic cells observed that absorbed dye and did not appear to be associated with crystals, but these were much fewer in number. Cell morphology changed in cells exposed to oxalate. There was a dose-dependent increase in intracellular vacuoles and apoptotic bodies. Both calcium oxalate monohydrate and dihydrate crystals were observed to form in chambers treated with oxalate. The crystals seemed to be fairly adherent to cells (or the well/chamber bottom), as movement, washing, or other manipulation generally failed to remove them. Cells associated with or close to crystals did not appear to have any increase in morphologic change. Despite decreased membrane integrity at shorter time periods (1–2 h), cell loss with crystal islands was not observed at 24 or 48 h.

Oxalate has been shown to induce proliferation, apoptosis, and necrosis in cortical renal epithelial cells from the proximal and distal convoluted tubules in a time- and concentration-dependent manner. These cell lines experience toxicity at concentrations of oxalate that are far greater than the theoretical oxalate load at the respective location in the nephron. Initiation of stone formation is not believed to occur in these anatomic areas of the kidney, but in the interstitium or tubular structures within the renal papilla. The interstitium is an attractive location for the initial nucleation and growth of kidney stones because of its relatively stagnant fluid physiology, but not all stones appear to form from a classic Randall's plaque.

The concentration of sodium oxalate required to demonstrate cytotoxic effects in IMCD cells was higher than that demonstrated in cortical epithelial cells by 5-fold. Concentrations of oxalate commonly experienced by IMCD cells likely closely resemble the urinary oxalate concentration of 0.2–1 mM. Proximal cortical epithelial cells experience oxalate concentrations similar to, or slightly higher than plasma (less than 3 μ M), and distal tubular oxalate concentrations are believed to be less than 50 μ M, although the older studies that these numbers were based on likely did not have the sensitivity available in recently developed assays. Other studies have shown LLC-PK1 (proximal) cells to have net cell loss at concentrations greater than 0.4 mM and MDCK (distal) cells to have net cell loss greater than 1 mM. Thus, IMCD cells commonly experience oxalate concentrations that are toxic to cortical cells but are themselves below the threshold necessary for cell loss. Toxic concentrations of

oxalate can be achieved in disease states such as primary hyperoxaluria and may be achieved in some instances of enteric/dietary hyperoxaluria. A low urinary output state may increase oxalate concentrations in the tubule, making toxicity possible.

Crystal–cell interaction appeared to play at least some role in oxalate toxicity, as trypan blue staining demonstrated a higher proportion of cells with membrane permeability in the immediate vicinity of or in direct contact with spontaneously formed calcium oxalate monohydrate crystals. Although not investigated in this particular study, this finding raises an interesting point regarding the relative importance of the toxic manifestations of calcium oxalate monohydrate crystals versus free oxalate. Crystal formation could be the initiator of cell loss. Another possibility is that crystals formed at sites where oxalate ion caused cellular damage, as adherence is more commonly observed in cells that have been damaged or lost polarity. To explain the experimental observation of clusters of damaged cells, however, either one affected cell causes the loss of membrane integrity in nearby cells or the adherence and formation of a crystal cause local cell damage from a “bystander” effect.

Free radical generation from oxalate exposure was an interesting observed phenomenon. The time- and concentration-dependent increase suggests that cellular reparative and reactive oxidative scavenging mechanisms are overwhelmed at higher oxalate concentrations. It is thought that ROS production initiates a series of intracellular events that culminate in increased membrane permeability, which variably leads to cell survival or death. The data demonstrate that the cells that are able to tolerate a certain amount of oxidative stress without cell loss at concentrations less than 2 mM did have increased production of free radicals. Trypan blue staining also demonstrated increased nuclear uptake in direct relation to oxalate exposure, even at lower oxalate concentrations.

Cell death in the inner medulla could lead to areas of exposed basement membrane along the tubule, which could provide areas of crystal attachment and growth. Additionally, disrupted epithelial tight junctions could allow access for concentrated lithogenic molecules in urine into the interstitium. Most patients with recurrent nephrolithiasis due to high oxalate excretion undoubtedly have a very low ratio of stones formed in the several hundred collecting ducts, but perhaps even a subtle change in the environment experienced by the renal papilla could slightly change this ratio, resulting in substantially more or fewer stones.

Perhaps cell loss from higher concentrations of oxalate is a protective mechanism. Oxalate crystals have been shown to attach to IMCD cells. Rapid cell death and cell sloughing may encourage the excretion of attached crystals that would later form larger stones. This could be initiated by higher oxalate concentrations rather than crystal attachment and could serve to excrete attached crystals of any type. This suggests that a degree of hyperoxaluria sufficient to cause epithelial toxicity would generate a much larger number of stones without this protective mechanism. In other words, stone formers may have a defect in the ability of IMCD cells to slough or may have some factor that reduces re-epithelialization after cell loss (i.e., dehydration or acidosis).

With idiopathic calcium oxalate urolithiasis likely being a multifactorial disease, it is unlikely that any one cause of this disease can be found. Additionally, the biological role of physiologic processes can be difficult to discern from *in vitro* studies, as local cell death could be beneficial or detrimental with regard to crystalluria and stone formation. The threshold for oxalate toxicity in mouse IMCD cell lines is much higher than in other renal epithelial cell lines, which correlates with the urinary milieu these cells commonly experience.

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FIGURE 1.

Photographs of IMCD cells exposed to oxalate. (A) Ninety-six-hour control (200 $\times$, hematoxylin and eosin). (B) Ninety-six-hour 1 mM sodium oxalate (200 $\times$, hematoxylin and eosin). (C) Ninety-six-hour 4 mM sodium oxalate (200 $\times$, hematoxylin and eosin). (D) Two-hour control with trypan blue (400 $\times$, eosin). (E) Two-hour 1 mM sodium oxalate with trypan blue demonstrating staining without crystals (400 $\times$, eosin). (F) Two-hour 4 mM sodium oxalate with trypan blue demonstrating staining with crystals nearby (400 $\times$, eosin).