Urol Res (2003) 31: 198–206
DOI 10.1007/s00240-003-0317-2

ORIGINAL ARTICLE

**Shizuka Iida · Masaru Ishimatsu · Shyumei Chikama
Michiro Inoue · Kei Matsuoka · Takashi Akasu
Shinshi Noda · Saeed R. Khan**

Protective role of heparin/heparan sulfate on oxalate-induced changes in cell morphology and intracellular Ca^{2+}

Received: 8 August 2002 / Accepted: 20 March 2003 / Published online: 29 April 2003

Abstract Alterations in intracellular Ca^{2+} ($[Ca^{2+}]_i$) are generally associated with cellular distress. Oxalate-induced cell injury of the renal epithelium plays an important role in promoting CaOx nephrolithiasis. However, the degree of change in intracellular free calcium ions in renal epithelial cells during oxalate exposure remains unclear. The aim of this study is to determine whether acute short-term exposure to oxalate produces morphological changes in the cells, induces a change in cytosolic Ca^{2+} levels in renal tubular epithelial cells and whether the application of extracellular glycosaminoglycans (GAGs) prevents these changes. Cultured Mardin-Darby canine kidney cells were exposed to oxalate, and changes in cytosolic Ca^{2+} were determined under various conditions. The effect of heparin and heparan sulfate (HS) during oxalate exposure was examined. The change in the GAG contents of the culture medium was also determined. Transmission electron microscopy (TEM) was performed for morphological analysis. The degree of change in cytosolic Ca^{2+} strongly correlated with oxalate concentration. Cytosolic Ca^{2+} levels decreased in parallel with an increase in the concentration of oxalate. However, this decrease was strongly inhibited by pretreatment with heparin or HS. TEM revealed cytoplasmic vacuolization, the appearance of flocculent material and mitochondrial damage after oxalate exposure. On the other hand, pretreatment with heparin or HS completely blocked these morphological changes. The present data suggest that acute exposure to a high concentration of oxalate challenges the renal cells, diminishes their viability and induces changes in cytosolic Ca^{2+} levels. Heparin and HS, which are known as potent inhibitors of CaOx crystallization, may also prevent oxalate-induced cell changes by stabilizing the cytosolic Ca^{2+} level.

Keywords Calcium oxalate · Intracellular Ca^{2+} · MDCK cells · Heparin · Heparan sulfate

S. Iida (✉) · S. Chikama · M. Inoue · K. Matsuoka · S. Noda
Department of Urology,
Kurume University School of Medicine,
67 Asahi-machi, Kurume 830–0011, Japan
E-mail: iidas@med.kurume-u.ac.jp
Tel.: +81-942-317572
Fax: +81-942-342605

M. Ishimatsu · T. Akasu
Department of Physiology,
Kurume University School of Medicine,
Kurume, Japan

S. R. Khan
Department of Pathology and Laboratory Medicine,
University of Florida College of Medicine,
Gainesville, USA

Introduction

Calcium oxalate (CaOx) is a major constituent of calcium-containing kidney stones. However, the mechanism underlying the formation of CaOx stones remains unclear. Recent studies indicate that a crystal-cell interaction is an important step during microlith formation [17, 25, 34]. It is believed that uncomplicated crystalluria alone does not initiate kidney stone disease. Finlayson and Reid [7] reported that under the most favorable conditions it takes 10 h for a 1-μm CaOx crystal to grow large enough to block the duct of Bellini and become the nidus of a urinary stone. Because the urinary transit time from glomerulus to the renal pelvis is approximately 3 min, crystalline particles formed in urine flowing freely through the renal tubule do not stay in the lumen long enough to attain the dimensions required to block a collecting duct and form microliths [7, 20]. These observations suggest that a more complicated mechanism is responsible for the development of clinical nephrolithiasis. It has been suggested that oxalate, which is an end product of metabolism and secreted in urine, can induce renal cell injury [17, 21, 28, 35], promoting crystal attachment and retention within the renal tubules. Several researchers have reported that

heparin or heparan sulfate (HS) inhibit calcium oxalate crystal aggregation, as well as crystal adhesion to the renal epithelial cells [5, 30, 36]. Since changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) are a hallmark of injury, we examined changes in intracellular free calcium ions in renal tubular epithelial cells during oxalate exposure. In this study, the cells were exposed to various concentration of oxalate under Ca^{2+}-free conditions in order to study the effect of oxalate ions on cell behavior without the complicating consequence of calcium and oxalate complexing and crystal formation. We also examined the effect of heparin and HS on cytosolic Ca^{2+} in the same manner as described above.

Materials and methods

Cell culture

Mardin-Darby canine kidney (MDCK) cells (ATCC CCL 34; Laboratory Products Division, Dainippon Pharmaceutical, Osaka, Japan) were subcultured in minimum essential medium (MEM; Gibco, N.Y., USA) containing 10% fetal calf serum (FCS; Gibco) and 1% antibiotics (penicillin and streptomycin)/antimycotic solution (Gibco) at 37°C in a 5% CO_2 and 95% air atmosphere. For additional experiments, 3×10^5 cells (passage 60–75) were seeded on to a 35 mm diameter glass-bottom dish (MatTek, Ashland, Mass., USA) and incubated overnight in an atmosphere of 5% CO_2/95% air in a humidified 37°C incubator in MEM supplemented with fetal calf serum and antibiotics. We used the confluent state MDCK cells in a preliminary experiment, and noticed that a high nucleus/cytosol ratio of the confluent state cells limited cytosolic Ca^{2+} measurement by the interference of Ca^{2+} fluorescence from the nucleus region. In this study, we used, in most cases, subconfluent MDCK cells that were grown up to 60–70% confluence to evaluate the intracellular calcium level.

Preparation of cells for intracellular Ca^{2+} assay

To measure cytosolic Ca^{2+}, cells were prepared using a membrane permeable intracellular Ca^{2+} indicator, Oregon Green BAPTA-1 AM (Molecular Probes, Ore., USA). The cells were washed twice in normal tyrode (NT) buffer (135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM NaOH-HEPES, 10 mM glucose, pH 7.4). Then, NT buffer containing Oregon Green 488 BAPTA-1 AM was added. The contents of the dye solution were as follows: 50 μg of Oregon Green was first dissolved in 4 μl DMSO and 2 μl of 20% pluronic acid (Molecular Probes, Ore., USA) and then diluted with normal tyrode (NT) solution to a final concentration of 10 μM (0.001% v/v). The cells were treated with 1 ml of dye solution for 30–60 min at room temperature.

Measurement of cytosolic Ca^{2+}

Fluorescent images of dye-loaded cells were captured using a fluorescent microscope system. After replacing the dye solution with 1 ml of NT solution, a culture dish was mounted on the stage of an inverted fluorescent microscope (Diaphotot 300, Nikon, Tokyo, Japan) equipped with a calcium measurement system (ARGUS-50, Hamamatsu Photonics, Hamamatsu, Japan). MDCK cells were illuminated by an excitation light with a 490-nm wavelength, and emission fluorescence (>515 nm) was captured through a barrier filter by intensified CCD camera (C2400-87, Hamamatsu Photonics). Eight to 16 fluorescent images were captured at a video rate, and the images were averaged into one image. An averaged image was taken every 10 s for 20–30 min to create sequential images. In this way, a remarkable decline in the baseline due to dye quenching was prevented. Two or three square boxes of 10×10 pixels on the cytosolic region were marked on a computer monitor and the fluorescent intensity of each box was measured on line. Since the initial value of intensity varied among cells, $\Delta F/F0$ was used, where F0 was the mean resting value obtained in the initial 60 s excluding the rapid rising phase.

Application of experimental solutions

To evaluate the degree of change in cytosolic Ca^{2+} under various conditions, we altered the concentration of sodium oxalate (0.5–2.0 mM) in calcium nominally free solution, the composition of which is the same as that of NT solution without calcium chloride. There was no effect on the osmotic pressure after the elimination of calcium from the NT solution. Moreover, we confirmed that the addition of sodium oxalate had no effect on the pH level of the calcium free solution (pH 7.4). Each test solution was applied to a culture dish (controlled to a total volume of 100 μl) by a continuous flow system (flow speed: 1.5 ml/min) and kept at 37°C. In each experiment, a minimum of four replicates were performed for each treatment. In some experiments, MDCK cells were pretreated with either heparin (final concentration 0.1–10 U/ml, Nakarai Tesque, Tokyo, Japan) or HS (final concentration 0.001–0.1 mg/ml, Seikagaku Kogyo, Tokyo, Japan) for 5 min by addition to culture dishes just before the Ca^{2+} measurement. Then the cells were rinsed with NT solution and evaluated for sequential Ca^{2+} measurement.

Measurement of extracellular calcium ions

To evaluate the effect of oxalate exposure on the extracellular calcium ion level, the concentration of free calcium ions was measured before and after oxalate exposure. MDCK cells were grown in 25 cm^2 flasks either to the 70% confluent or to the confluent phase. The cells were rinsed with D-PBS solution twice and then either 5 ml of Ca^{2+}-free NT solution or oxalate containing Ca^{2+}-free NT solution was added. Cells were incubated for 30 min at 37°C and the supernatant was collected to measure the calcium ion level. The changes in extracellular calcium ion levels between the control group and oxalate-exposed groups were examined by using ion-selective electrode methods at a clinical testing laboratory (SRL, Tokyo, Japan).

Transmission electron microscopy

For TEM, MDCK cells were grown in 35 mm diameter Falcon culture dishes (EASY GRIP, Becton Dickinson, N.J., USA) to 60–70% confluence. After being washed three times in NT solution, the cells were incubated in a calcium-free solution containing 2 mM sodium oxalate at 37°C for 15 min in a CO_2 incubator. Pretreatment of HS or heparin was also performed, and the cells were then incubated in the same manner as described above. Then, the buffer was aspirated and the cells were fixed with 1.0% glutaraldehyde for 1 h, before being treated with 1% osmium tetroxide for 30 min, dehydrated in a graded ethanol series, and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). Ultra thin sections were stained by lead citrate for 7 min, and these were examined using a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan). All chemicals were of the highest grade available.

Measurement of cell viability

To assess cell viability in the presence or absence of oxalate, an MTT assay was performed [27]. In brief, MDCK cells (4×10^3 cells/well) were seeded on a 96-well-plate (Falcon) and cultured for 24 h.

They were then used in the following experiment. Cells were incubated in 100 μl of Ca^{2+} free solution, with or without NaOx (0.5–2 mM), for 30 min. For each plate, absorbance was monitored using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell growth assay kits (Chemicon, Temecula, Calif., USA), according to the manufacturer's instructions. The number of viable cells was estimated by measuring the absorbance of each well with Easy Reader EAR 400 (SLT Lab Instruments, Salzburg, Austria). The rates of viable cells were obtained as the ratio of experimental absorbance (viable cell number in the experimental wells) to control absorbance (viable cell number in the control wells).

Measurement of total GAGs concentration in the medium after oxalate exposure

To assess the change of total GAGs levels after oxalate exposure, the total GAGs concentration was quantified with a modified dimethylmethylene blue (DMB) method [6, 13, 26].

In brief, MDCK cells were grown in 25 cm^2 flasks to confluence and then rinsed with D-PBS solution twice. Then either 5 ml of Ca^{2+}-free NT solution or oxalate containing Ca^{2+}-free NT solution was added. Cells were incubated for 30 min at 37°C and the supernatant collected to measure the total GAGs level. A total of 10 μl medium was collected and 90 μl of DMB solution was added before detection at 535 nm using a microplate reader (Bio-Rad) [13, 26].

Statistical analysis

Data are expressed as mean ± SE. Differences between data sets were analyzed by either the unpaired *t*-test or the Mann-Whitney U-test. $P < 0.05$ was considered to be statistically significant.

Results

Effect of oxalate on intracellular calcium in MDCK cells

The concentration of free oxalate was measured in calcium nominally free solution containing 0.5–2 mM sodium oxalate using a capillary electrophoresis system at a clinical testing laboratory (SRL). The concentration of oxalate used in the present study was 0.5–2 mM (0.31–1.51 mM as free oxalate). To examine the effect of oxalate on $[Ca^{2+}]_i$, MDCK cells were subjected to various concentrations in a nominally calcium-free solution, since oxalate easily binds to extracellular free calcium and this results in crystal formation in the extracellular space. At 3 min after adding the oxalate (2 mM) to the test solution, calcium fluorescence in the cytoplasmic region turned pale and the intracellular calcium level reduced sharply to 67.8 ± 5.7% ($n = 4$) of the initial value (Fig. 1A–C). The reduction in the calcium level did not recover over an observation period of 30 min after removal of the oxalate (data not shown).

To exclude the possible direct effect of a nominally calcium-free solution on $[Ca^{2+}]_i$, the period of calcium free solution was extended to 8 min prior to oxalate application. Under such conditions, the calcium-free solution did not change the basal level of calcium fluorescence, however, oxalate again decreased $[Ca^{2+}]_i$ to

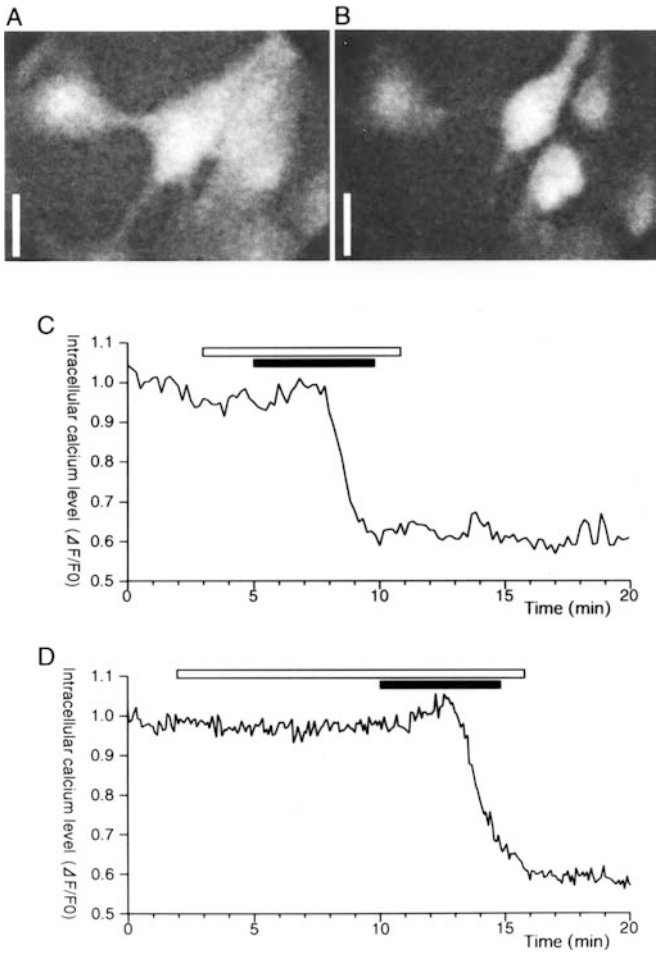

Fig. 1 Oxalate exposure diminished $[Ca^{2+}]_i$ in MDCK cells. Calcium fluorescent images of the cells **A** before and **B** 5 min after addition of oxalate. Calcium fluorescence of the cytosolic lesion is remarkably reduced, while fluorescence of the nucleus is unchanged. The *scale bar* denotes 20 μm. In the *middle* and *bottom* panels, oxalate (2 mM) perfused during the period indicated by a *solid bar* in a nominally calcium-free solution (*open bar*). **C** $[Ca^{2+}]_i$ is reduced by oxalate. **D** Extended period in a nominally calcium-free solution did not affect $[Ca^{2+}]_i$ while oxalate immediately lowered $[Ca^{2+}]_i$

63.2 ± 0.07% ($n = 3$) accompanied by the same degree of delay time (Fig. 1D). We extended the calcium free period up to 20 min and confirmed that the calcium free solution itself had no remarkable effect on $[Ca^{2+}]_i$ during the observation period (Fig. 1D). We also tested lower concentrations of oxalate on $[Ca^{2+}]_i$ in MDCK cells. While 1 mM was less effective than 2 mM oxalate, it still reduced $[Ca^{2+}]_i$ (69.3 ± 5.9%, $n = 5$) significantly. Although the reduction of $[Ca^{2+}]_i$ could be seen at a 0.5 mM oxalate challenge, it was not significant (Fig. 2A).

We also examined the effect of oxalate on the state of confluence of the MDCK cells. While the large nucleus/cytosol ratio made it difficult to measure the cytosolic calcium level, the change of $[Ca^{2+}]_i$ was significantly decreased after 1 mM oxalate exposure (86.4 ± 5.1%, $n = 6$, Fig. 2A). Since we were interested in the exact

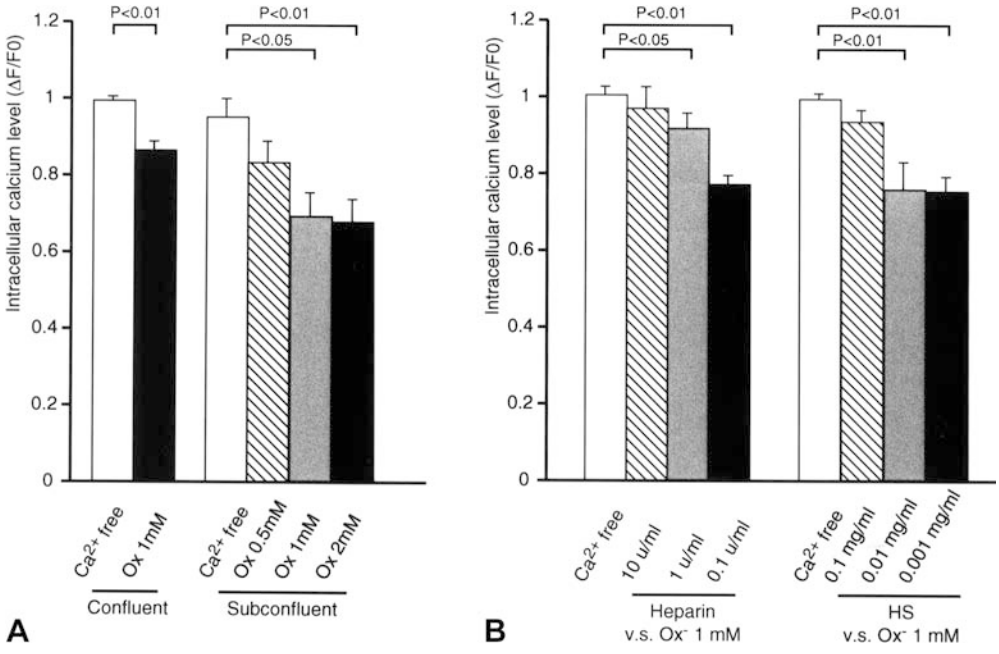

Fig. 2 Change of $[Ca^{2+}]_i$ in each group before and after oxalate challenge. **A** In a control condition, oxalate (1 mM) significantly reduced $[Ca^{2+}]_i$ to $86.4 \pm 2.1\%$ ($P < 0.01$) in confluent state MDCK cells. Such an effect of oxalate was remarkable in sub-confluent state MDCK cells. Cytosolic Ca^{2+} levels decreased significantly after 1 mM and 2 mM of oxalate exposure ($P < 0.05$, $P < 0.01$, respectively). **B** Pretreatment either with heparin or HS showed a preventive effect against oxalate (1 mM) exposure

change in cytosolic calcium level, we used sub-confluent MDCK cells in this study.

Preventive effect of heparin/heparan sulfate on the decline of $[Ca^{2+}]_i$ during oxalate exposure

Both heparin and heparan sulfate are known to have a preventive effect on CaOx crystal adhesion to renal epithelial cells [5, 30, 36]. In the present study, we examined whether or not these substances have an effect on cytosolic free Ca^{2+} during oxalate challenge. The upper panel of Fig. 3 shows the effect of oxalate (2 mM, 5 min) on HS pretreated MDCK cells. The intensity of calcium fluorescence in the cytosolic region was little affected, like the nuclear region, unlike cells without HS treatment. Pretreatment with heparin also prevented MDCK cells from $[Ca^{2+}]_i$ reduction (Fig. 3). Various concentrations of HS (0.1–0.001 mg/ml) or heparin (10–0.1 U/ml) were used as a pretreatment for 5 min before oxalate exposure. Both heparin and HS prevented the effect of oxalate on $[Ca^{2+}]_i$ when a high concentration was used, but such preventive effects were lost in a dose dependent manner (Fig. 2B).

The changes of extracellular Ca^{2+} during oxalate exposure

To assess whether or not intracellular calcium ions leak out the extracellular space after oxalate exposure, extracellular calcium ions were measured. Figure 4 shows the change of extracellular calcium ion levels between the control group (nominally Ca^{2+} free solution) and the oxalate-exposed (0.5 mM and 1 mM) groups. There was no significant difference between the control

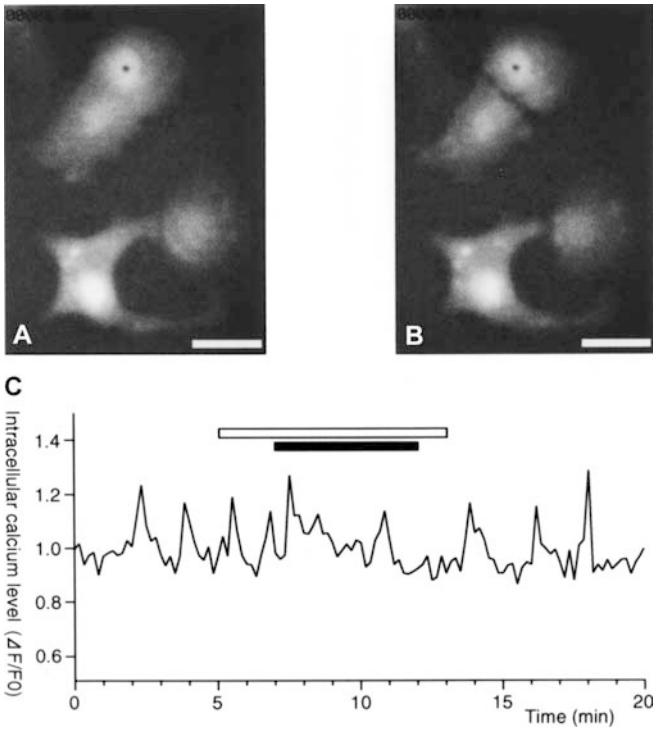

Fig. 3 HS prevented the effect of oxalate on $[Ca^{2+}]_i$. MDCK cells were treated by HS (0.1 mg/ml) for 5 min and calcium fluorescent images were captured before and 10 min after perfusion of oxalate (**A**, **B**). Note that a large cytosolic area remained after prolonged exposure of oxalate. The *scale bar* denotes 20 μm. **C** Perfusion of oxalate did not affect $[Ca^{2+}]_i$ on HS treated cells. Data were obtained from distinct cells shown in **A** and **B**

and oxalate-exposed groups ($n = 6$ each). To exclude the possibility of the consumption of calcium ions via the precipitation as calcium oxalate crystals, light microscopy was performed. No crystals were detected in the

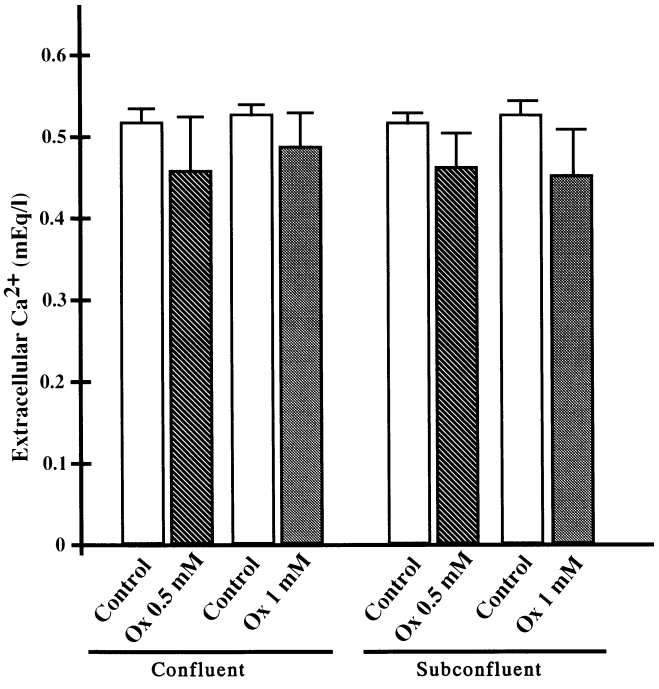

Fig. 4 The change of extracellular calcium ion levels between the control group (nominally Ca^{2+} free solution) and oxalate-exposed (0.5 mM and 1 mM) groups. There is no significant difference between the control and oxalate-exposed groups. (1 mEq/l = 0.5 mmol/l)

medium (data not shown). These observations strongly suggest that intracellular calcium ions did not leak out to the extracellular space after oxalate exposure.

Morphological structure of MDCK cells after oxalate exposure

To evaluate the morphological changes of cells after oxalate exposure, TEM was performed. Figure 5A shows the normal morphological structure of MDCK cells (×10,000). TEM revealed cytoplasmic vacuolization, aggregation of flocculent entities and cellular debris (arrow head) after 15 min. of 2 mM oxalate exposure (Fig. 5C, D, ×8,900, ×24,000, respectively). Moreover, injured mitochondria (arrow) were seen near the cytoplasmic vacuoles (Fig. 5E, F, ×6,600, ×21,000). These observations were made ubiquitously in the oxalate exposed cells. On the other hand, heparin pretreatment inhibited cell damage and morphological change (Fig. 5B, ×12,000). HS-treated MDCK cells showed a similar construction to those with heparin treatment (data not shown).

Oxalate induced decrease in cell viability

To evaluate the viability of the oxalate exposed cells, an MTT assay was performed. The number of viable cells significantly decreased within 1 h during 2 mM oxalate exposure ($P < 0.01$) (Fig. 6). However, a challenge with 1 mM oxalate was less effective on the decline of cell viability ($P < 0.05$). On the other hand, cells pretreated by either heparin or HS were protected against the decline in the number of viable cells under 1 mM oxalate exposure (Fig. 6). These observations indicate that heparin and HS prevent the cell injury by oxalate.

Oxalate induced increase of total GAGs levels

The concentrations of GAGs in the medium were determined in both normal control and oxalate exposed groups. Figure 7 shows the total GAGs concentration in each group measured by modified DMB methods [13, 26]. Oxalate exposure resulted in an increase in total GAG contents of the medium and reached a significant level following exposure to 1 mM oxalate (Fig. 7).

Discussion

In this report, we provide the first direct evidence that a high concentration of extracellular free oxalate can induce a change in cytosolic Ca^{2+} levels in renal epithelial cells. Moreover, the findings of both TEM and MTT assay reveal that acute exposure to higher concentrations of oxalate might injure the cells within a short time. Although the exact mechanism of the oxalate-induced change in cytosolic Ca^{2+} levels remains unclear, the following hypotheses should be considered. The first hypothesis is that extracellular oxalate ions are capable of entering through the plasma membrane to the cytosol where they bind with cytosolic Ca^{2+} to create an intracellular crystalline structure. Although we could not confirm the presence of any crystals in the cytosolic region after oxalate exposure, the TEM findings implied some important changes. The second hypothesis is that intracellular calcium leaked out from the cytosol following oxalate induced cell injury. If the injured cell membrane became leaky, intracellular calcium ions may come out in the minimal calcium environment. To confirm this hypothesis, we measured the extracellular calcium ions in both normal control and oxalate exposed groups. However, there was no significant difference between the control and oxalate exposed groups (Fig. 4). The third hypothesis is that intracellular calcium is returned to intracellular calcium stores such as mitochondria and endoplasmic reticulum. Even though we have not localized calcium stores inside the cells, flocculent material may represent calcium bound to cytosolic proteins.

Both the decline in intracellular $[Ca^{2+}]_i$ as well as cell viability were dramatically inhibited by the presence of either heparin or HS in a dose dependent manner. These phenomena imply that heparin and HS prevent oxalate induced-cell injury or may also prevent oxalate from entering the cells via the creation of a charge barrier. In the kidney, HS is the major acid GAG constituent of

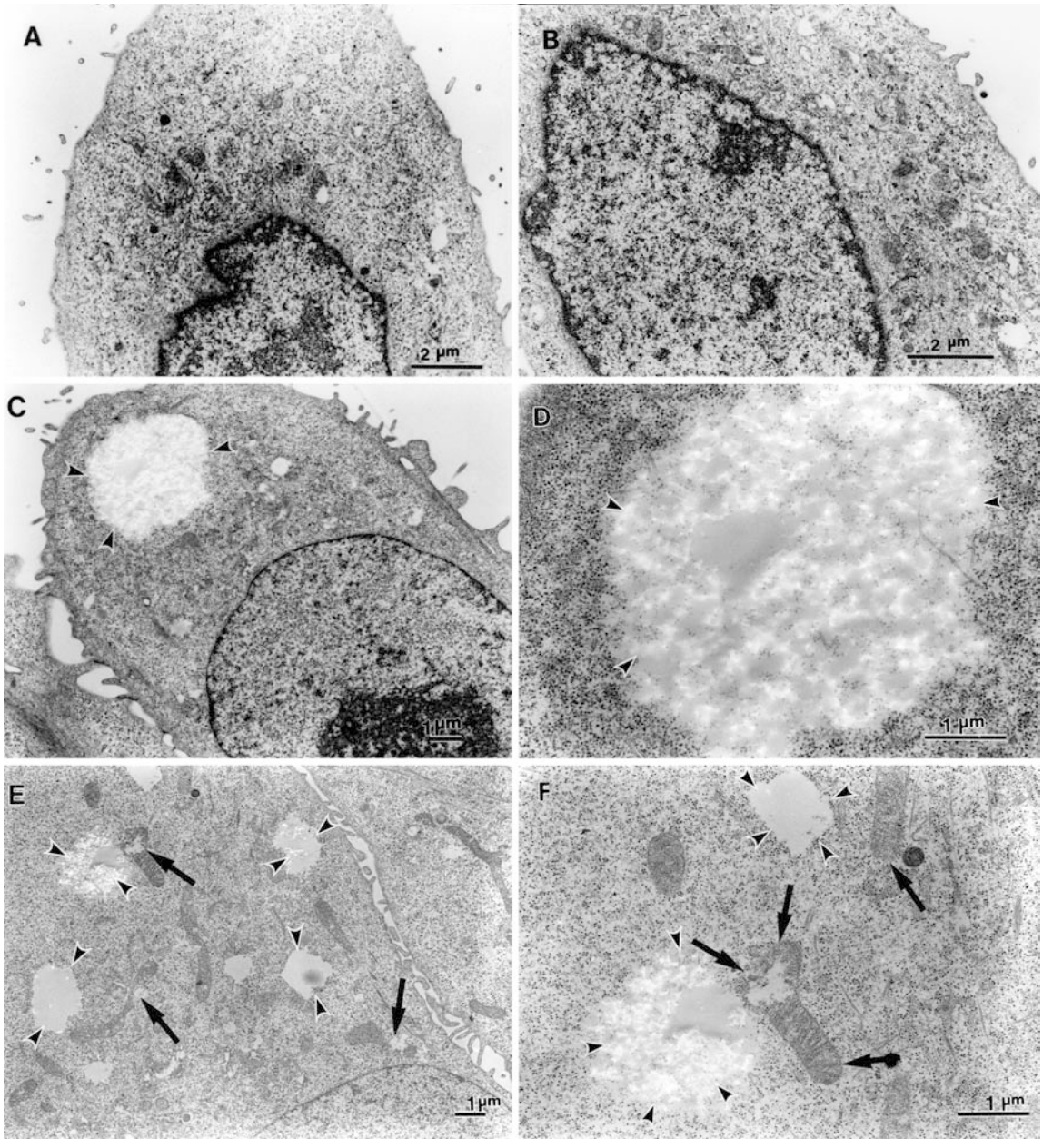

Fig. 5 The morphological effect on MDCK cells after oxalate exposure. To evaluate morphological changes in the cells after oxalate exposure, transmission electron microscopy was performed. **A** shows the normal morphological structure of MDCK cells (magnification ×10,000). **B** shows the structure of heparin-treated cells after 15 min of 2 mM oxalate exposure (magnification ×12,000). Heparin prevented cell damage caused by oxalate challenge. **C**, **D** On the other hand, in oxalate-exposed MDCK cells, cytoplasmic vacuolization and the accumulation of flocculent material (*arrow heads*) was observed after 15 min of oxalate exposure (magnification: ×8,900, ×24,000, respectively). **E**, **F** In the other part of oxalate-exposed cells, the break down of the mitochondrial structure was observed (*arrows*). The *arrowheads* indicate cytoplasmic vacuolization and the accumulation of flocculent material (magnification ×8,600, ×21,000, **E** and **F**, respectively)

both the glomerular and tubular basement membranes [12]. In general, GAGs play an important role in crystal-cell interactions. Removal of GAGs from uroepithelial cell surfaces promotes crystal adhesion and the process is recovered by treatment with sulfated polysaccharides, such as heparin and chondroitin sulfate [8]. Pretreatment of CaOx crystals with sulfated GAGs prevents their adherence to LLC-PK1 and MDCK cells in culture [33]. Heparin also inhibits endocytosis of CaOx crystals on BSC-1 monkey kidney epithelial cells via an interaction with cells and not with crystals [23]. These observations support the hypothesis that cell-surface GAGs provide a protective barrier against crystals and oxalate ions.

Interactions between renal epithelial cells and CaOx crystals (or oxalate ions) no doubt play a crucial role in the formation of urinary stones [17, 18]. Renal epithelial cells respond to hyperoxaluria and the presence of CaOx crystals both in vivo [9, 16] and in vitro [10, 24]. It is well known that calcium-binding proteins as well as GAGs

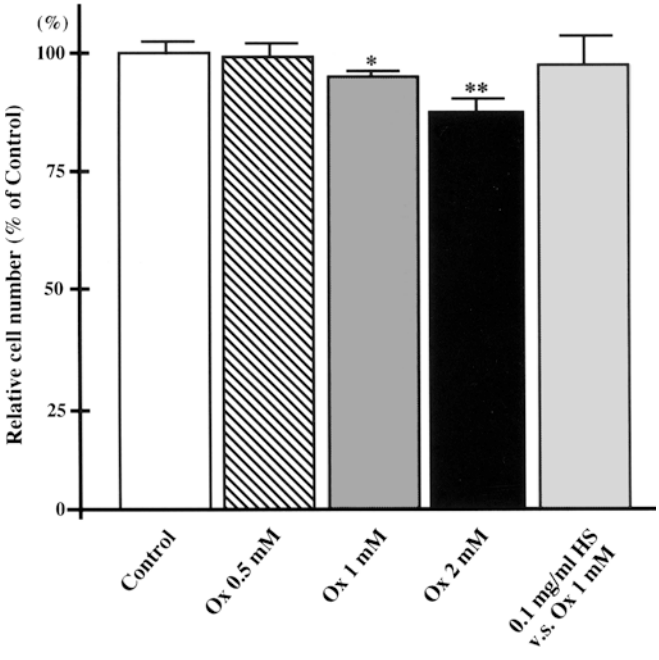

Fig. 6 To evaluate the viability of the oxalate exposed cells, MTT assay was performed. Each group contained four wells and this experiment was repeated four times compared with control wells. (*$P < 0.05$, **$P < 0.01$)

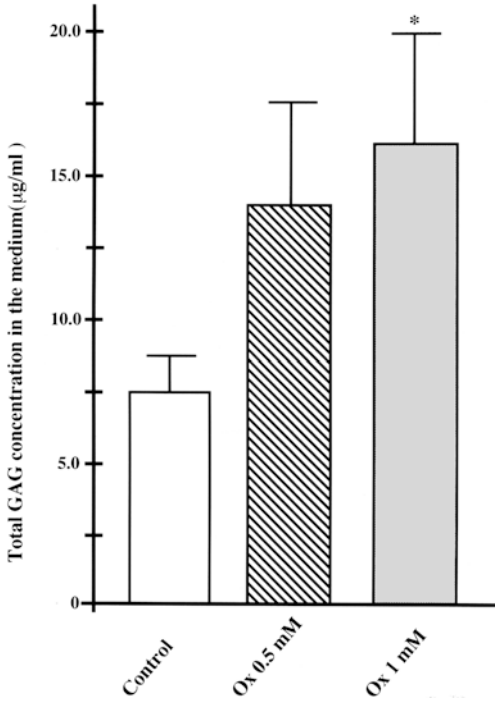

Fig. 7 The concentrations of GAGs in the medium determined in both normal control and oxalate exposed cells. Total GAGs concentration in each group measured by modified DMB methods. ($n = 6$ each, *$P < 0.05$)

chelate calcium and coat the surfaces of calcium containing crystals, which are then either excreted as crystaluria particles or endocytosed by the epithelial cells. Crystals produced in such an environment have protein coats and are apparently less reactive with renal epithelial cells [18]. Our previous study implied that CaOx nephrolithic conditions might induce increased expression of HSPG in the tubular epithelial cells and thereby protecting and repairing the damaged epithelial surface [14, 15]. Moreover, Barsotti and colleagues reported on the effect of HS administered in a rat remnant kidney model [2]. In their study, HS-treated rat kidney showed a lower prevalence of glomerular sclerosis, mesangial proliferation, and much less evident tubulointerstitial damage than the controls. They concluded that HS might act as a renoprotective substance via anti-proliferative and anti-inflammatory effects [2].

Oxalate, which is a common constituent of kidney stones, induces renal injury [17, 19, 21, 28]. Moreover, a high concentration of both oxalate and COM crystals also induces the expression of the early-response gene in renal epithelial cells [10, 22]. In general, increases in cytosolic Ca^{2+} are believed to be a pivotal signal in the regulation of cell injury, cell death, cell proliferation, cellular differentiation and cellular aging. Reversible cellular changes following injury include cellular swelling, cytoplasmic bleb formation, mitochondrial condensation or swelling, nuclear chromatin clumping, dilation of the endoplasmic reticulum with a scattering of polysomes, and the formation of autophagic vacuoles, resulting ultimately in secondary lysosomes [31]. On the other hand, lethal cell injury induces mitochondrial swelling, fragmentation of the nucleus, and karyolysis [31]. Increased cytosolic Ca^{2+} initiates the activation of calcium-dependent endonucleases, a process that is followed by DNA strand breakage and cell death [4, 29, 31, 32]. Mitochondrial inclusions are precipitates of mitochondrial proteins in the form of dense flocculent aggregates, often referred to as flocculent densities, and precipitates of calcium phosphate which typically later become crystalline hydroxyapatite [31]. The exact mechanism underlying the intracellular crystallization caused by oxalate injury remains unclear. Scheid and colleagues reported on the measurement of cytosolic Ca^{2+} in LLC-PK1 cells, which originate from porcine renal proximal tubules during challenge with high concentrations of oxalate [28, 37]. They concluded that a high concentration of oxalate ions induces renal cell injury. However, oxalate challenge had little or no effect on the resting levels of either $[H^+]_i$ or $[Ca^{2+}]_i$ [28]. In our studies, while the final concentration of free oxalate was higher than in a previous report [28], it was still within the range of primary hyperoxaluria [1]. Moreover, even in healthy individuals, urinary excretion of oxalate can reach a primary hyperoxaluria level soon after the consumption of a high oxalate diet. The concentrations of sodium oxalate used by Scheid et al. were 0.1, 0.2, 0.4, 1.0, 2.0 and 4.0 mM (the free oxalate levels ranged in concentration from 0.03 to 1.6 mM) [28]. Since they added the sodium oxalate into the calcium-containing buffer, a part of the free oxalate ions was

consumed by forming calcium oxalate crystals. On the other hand, the concentration of oxalate used in the present study was 0.5–2.0 mM (0.31–1.51 mM as free oxalate). This discrepancy was likely caused by our use of Ca^{2+}-free buffer during oxalate exposure. In biological fluids, soluble oxalate can not exceed approximately 0.1 mM because at higher concentrations it will inevitably precipitate [3]. These levels seem to be somewhat higher than those under normal conditions. Hautmann [11] reported that the oxalate content of rat renal cortex was approximately 0.3 mM and the level of oxalate in the papilla was up to 1.0 mM. Although the exact mechanism underlying the first step of CaOx crystal formation remains unclear, the present data strongly suggest the possibility of oxalate induced changes in cell membranes and cytosolic Ca^{2+}. In this study, we explored the acute effect of oxalate on cytosolic calcium ion of MDCK cells. In a future study, cells should be exposed to lower concentrations of oxalate for prolonged times to estimate the influence of chronic mild hyperoxalic conditions.

Acknowledgments This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists (S.I., M.I.:11770915, 11770916), (S.N.: 12307072) from the Ministry of Education, Science, Sports, and Culture in Japan. We thank Ms. Satoko Yamada for her help in preparing the cells for the transmission electron microscopic study.

References

1. Balcke P, Zazgornick J, Sunder-Lassmann G, Kiss A, Hauser AC, Gremmel F, DerflerK, Stockenhuber F, Schmidt P (1989) Transient hyperoxaluria after ingestion of chocolate as a high risk factor for calcium oxalate calculi. Nephron 51: 32
2. Barsotti G, Cupisti A, Gervasi GB, Bartoli C, Barsotti M, Pasquariello A, Moriconi L, Giovannetti S (1999) Effects of oral administration of heparan sulphate in the rat remnant kidney model. Nephron 81: 310
3. Belliveau J, Griffin H (2001) The solubility of calcium oxalate in tissue culture media. Anal Biochem 291: 69
4. Dominguez JH, Song B, Liu-Chen S, Qulali M, Howard R, Lee CH, McAteer J (1996) Studies of renal injury II. Activation of the glucose transporter 1 (GLUT 1) gene and glycolysis in LLC-PK1 cells under Ca^{2+} stress. J Clin Invest 98: 395
5. Ebisuno S, Kohjimoto Y, Tamura M, Ohkawa T (1995) Adhesion of calcium oxalate crystal to Madin-Darby canine kidney cells and some effects of glycosaminoglycans or cell injuries. Eur Urol 28: 68
6. Farndale RW, Sayers CA, Barrett AI (1982) A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage culture. Connect Tissue Res 9: 247
7. Finlayson B, Reid S (1978) The expectation of free and fixed particles in urinary stone disease. Invest Urol 15: 442
8. Gill WB, Jones KW, Ruggiero KJ (1982) Protective effects of heparin and other sulfated glycosaminoglycans on crystal adhesion to injured urothelium. J Urol 127: 152
9. Gokhale JA, Glenton PA, Khan SR (1996) Localization of Tamm-Horsfall protein and osteopontin in a rat nephrolithiasis model. Nephron 73: 456
10. Hammes MS, Lieske JC, Spargo BH, Toback FG (1995) Calcium oxalate monohydrate crystals stimulate gene expression in renal epithelial cells. Kidney Int 48: 501
11. Hautmann RE (1977) Intra-renal calcium and oxalate concentration gradients. In: Rose GA, Robertson NG, Watts RWE (eds), Oxalate in human biochemistry and clinical practice. The Wellcome Foundation, London, p 147
12. Heuvel LPWJ, Born J, Veerkamp JH, Velden TJAM, Schenkels L, Monnens LAH, Schroder CH, Berden JHM (1990) Heparan sulfate proteoglycan from human tubular basement membrane. Comparison with this component from the glomerular basement membrane. Biochim Biophys Acta 1025: 67
13. Iida S, Suzuki K, Matsuoka K, Takazono I, Shimada M, Inoue M, Yahara J, Noda S (1997) Analysis of glycosaminoglycans in human prostate by high-performance liquid chromatography. Br J Urol 79: 763
14. Iida S, Miyajima J, Suzuki K, Matsuoka K, Inoue M, Noda S (1997) Expression of heparan sulfate proteoglycan mRNA in rat kidneys during calcium oxalate nephrolithiasis. Urol Res 25: 361
15. Iida S, Inoue M, Yoshii S, Yanagaki T, Chikama S, Shimada A, Matsuoka K, Noda S, Khan SR (1999) Molecular detection of heparan sulfate proteoglycan mRNA in rat kidney during calcium oxalate nephrolithiasis. J Am Soc Nephrol 10: S412
16. Iida S, Peck AB, Johnson-Tardieu J, Moriyama M, Glenton PA, Byer KJ, Khan SR (1999) Temporal changes in mRNA expression for bikunin in the kidneys of rats during calcium oxalate nephrolithais. J Am Soc Nephrol10: 986
17. Khan SR (1995) Calcium oxalate crystal interaction with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone development. Urol Res 23: 71
18. Khan SR (1997) Tubular cell surface events during nephrolithiasis. Curr Opin Urol 7: 240
19. Kohjimoto Y, Kennington L, Scheid CR, Honeyman TW (1999) Role of phospholipase A2 in the cytotoxic effects of oxalate in cultured renal epithelial cells. Kidney Int 56: 1432
20. Kok DJ, Khan SR (1994) Calcium oxalate nephrolithiasis, a free or fixed particle disease. Kidney Int 46: 847
21. Koul HK, Koul S, Fu S, Santosham V, Seikhon A, Menon M (1999) Oxalate: from crystal formation to crystal retention. J Am Soc Nephrol 10: S417
22. Koul H, Kennington L, Nair G, Honeyman T, Menon M, Scheid C (1994) Oxalate-induced initiation of DNA synthesis in LLC-PK1 cells, a line of renal epithelial cells. Biochem Biophys Res Commun 205: 1632
23. Lieske JC, Toback FG (1993) Regulation of renal epithelial cell endocytosis of calcium oxalate monohydrate crystals. Am J Physiol 264: F800
24. Lieske JC, Hammes MS, Hoyer JR, Toback FG (1997) Renal cell osteopontin production is stimulated by calcium oxalate monohydrate crystals. Kidney Int 51: 679
25. Lieske JC, Huang E, Toback G (2000) Regulation of renal epithelial cell affinity for calcium oxalate monohydrate crystals. Am J Physiol 278: F130
26. Moriyama M, Suzuki K, Nakajima C, Kawamura K, Miyazawa K, Tsugawa R (1994) Studies on the modified dimethylmethlene blue (DMB) method for determining glycosaminoglycans (GAG) in urine. Hinyokika Kiyo 40: 565
27. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55
28. Scheid C, Koul H, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Kennington L, Kohli R, Hodapp J, Avazian P, Menon M (1996) Oxalate toxicity in LLC-PK1 cells, a line of renal epithelial cells. J Urol 155: 1112
29. Smith MW, Phelps PC, Trump BF (1992) Injury-induced changes in cytosolic Ca^{2+} in individual rabbit proximal tubule cells. Am J Physiol 262: F647
30. Suzuki K, Ryall RL (1996) The effect of heparan sulfate on the crystallization of calcium oxalate in undiluted, ultrafiltered human urine. Br J Urol 78: 15
31. Trump BF, Berezesky IK (1992) The role of cytosolic Ca^{2+} in cell injury, necrosis and apoptosis. Curr Opin Cell Biol 4: 227

32. Ueda N, Shah SV (1992) Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells. J Clin Invest 90: 2593
33. Verkoelen CF, Romijn JC, Cao LC, Boeve ER, Bruijn WC, Schroder F (1996) Crystal-cell interaction inhibition by polysaccharides. J Urol 155: 749
34. Verkoelen, CF, van der Boom BG, Romijn JC (2000) Identification of hyluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. Kidney Int 58: 1045
35. Wiessner JH, Hasegawa AT, Hung LY, Mandel GS, Mandel NS (2001) Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells. Kidney Int 59: 637
36. Yamaguchi S, Yoshioka T, Utsunomiya M, Koide T, Osafune M, Okuyama A, Sonoda T (1993) Heparan sulfate in the stone matrix and its inhibitory effect on calcium oxalate crystallization. Urol Res 21: 187
37. Yanagawa M, Koul H, Honeyman T, Malhotra R, Scheid C, Menon M (1994) Oxalate-induced changes in intracellular calcium levels in renal papillary cells. In: Ryall R (eds) Urolithiasis 2. Plenum Press, New York, p 117