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Interactions between calcium oxalate monohydrate crystals and Madin-Darby canine kidney cells: endocytosis and cell proliferation

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Abstract The present investigation was designed to study the biological responses in cultures of Madin-Darby canine kidney (MDCK) cells exposed to calcium oxalate monohydrate (COM) crystals, the most common type of urinary crystals. The addition of COM crystals significantly accelerated the multiplication of MDCK cells and significantly activated the cell viability. After exposure of MDCK cells to COM crystals, scanning electron microscopy revealed that some crystals adhered to the plasma membrane and others were endocytosed by the cell. This cellular uptake of crystals was time dependent from 1 to 8 h and showed a specificity according to crystal type. However, the endocytosis of aggregated COM crystals was less marked than that of non-aggregated crystals. Pre-treatment with each of the glycosaminoglycans (sodium pentosan polysulphate, heparin, and chondroitin sulphate C) produced a significant reduction of the cellular uptake of COM crystals, suggesting that these glycosaminoglycans may play some critical roles in preventing the cellular uptake of crystals. Although investigation in further detail is necessary, we speculate that these crystal-cell interactions, that is, the cellular uptake of crystals and cell proliferation, may be among the earliest processes in the formation of kidney stones.

Key words MDCK · Calcium oxalate crystal · Crystal-cell interaction · Endocytosis · Cell proliferation · Glycosaminoglycans

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Introduction

The physicochemical theory of lithogenesis explains kidney stone formation by the precipitation, growth, and aggregation of several lithogenic salts in the urine [3]. However, the mechanism by which crystals form calculi in the kidney is still unclear. When urine flow is normal, the free calcium oxalate crystals formed within the renal tubules do not grow rapidly enough to block the collecting duct and become a kidney stone [2]. Therefore, it is likely that the crystals must be trapped in the tubular lumen or adhere to the epithelial cells for formation of kidney stones. Khan et al. [6] observed crystal attachment to the brush border of proximal tubules in rats. The relation between crystals and renal epithelial cells has been noted for a long time; for example, papillary casts and Randall's plaque hypothesis [15] has been known as a theoretical pathogenesis of human kidney stones.

However, these crystal-cell interactions have received little attention because the difficulties of an experimental evaluation would entail, although recent advances in cell biology techniques have led to the intensive investigation of these phenomena in some laboratories. Riese et al. [10, 16–18] reported the binding of calcium oxalate monohydrate (COM) crystals to rat renal papillary collecting tubule cells in primary culture. On the other hand, Lieske et al. noted engulfment of crystals into tubular epithelial cells and cell proliferation in a transplanted kidney in a patient with primary hyperoxaluria [8], and they confirmed this phenomenon experimentally using some calcium-containing crystals and tubular cells in culture [9]. However, neither the specific process of the crystal adhesion to the tubular cells nor the subsequent cellular response has been elucidated completely.

We are presently focusing on these crystal-cell interactions as important initiating events in nephrolithiasis and investigating these interactions between cultures of

Madin-Darby canine kidney (MDCK) cells and COM crystals using methods developed at our institution. MDCK cells exhibit many characteristics of the cortical collecting tubular cells of the kidney [4]. Recently, we reported that COM crystals adhered to intact MDCK cells in a time- and concentration-dependent manner, and that the presence of glycosaminoglycans and cell injuries reduced the attachments [1].

In the present study, we examined whether the most common urinary crystal, COM, provokes cell proliferation and engulfment of crystals in cultures of the canine kidney epithelial cells that are used as a model system to study crystal-cell interactions. We also studied the influence of certain other factors on these phenomena.

Materials and methods

Cell culture

MDCK cells (ATCC CCL 34) were obtained from the Laboratory Products Division of Dainippon Pharmaceutical Co. (Osaka, Japan) and subcultured in minimum essential medium (MEM; Gibco, New York, USA) containing 10% fetal calf serum (FCS; Nipro, Osaka, Japan) and 1% antibiotic (penicillin and streptomycin)/antimycotic (amphotericin B) solution (Gibco) at 37 °C in a 5% CO_2 and 95% air atmosphere. For the investigation of the effects of COM crystals on cell multiplication and the cellular uptake of crystals, 6×10^5 cells were plated in each well of the six-well culture plates (Nippon Becton Dickinson, Tokyo, Japan), and the nearly confluent cultures were used for study after 12 h. For the MTT assay, which is widely used to measure the activity of living cells dependent on the cleavage of the tetrazolium ring in active mitochondria [11], 3×10^5 cells were plated in each well of the 12-well culture plates, and the nearly confluent cultures were used for study after 12 h. Under phase contrast microscopy and scanning electron microscopy (SEM), most of the cells cultured in this manner contacted each other and showed no morphological differences from the cells in completely confluent cultures.

Crystals

Crystals of COM (Wako Pure Chemical Co., Osaka, Japan) and two other calcium-containing crystals, i.e., hydroxyapatite (HA; Nippon Chemical Co., Tokyo, Japan) and brushite (BR; Wako), were suspended in MEM and treated with ultrasound for 15 min in order to obtain uniform crystal conditions. SEM pictures verified the average sizes of these crystals, which were 0.7 (0.4–2.0) μm, 1.8 (0.8–6.5) μm, and 3.3 (1.2–6.9) μm, for COM, HA and BR, respectively. The treatment with ultrasound removed the aggregation of these crystals and did not alter the size and structure of the individual crystals.

In the experiments on the cellular uptake of crystals, COM crystals from three sources, i.e., Nacalai Tesque (Kyoto, Japan), produced in our laboratory, and Wako, were used. SEM examination verified the similar sizes of individual crystals from these three sources. Particle volume distribution curve measured with a TAII-Coulter counter demonstrated that these crystals differed in their degree of aggregation even after the treatment of ultrasound, with those from Nacalai Tesque, manufactured in our laboratory, and from Wako showing strong, moderate, and weak aggregation, respectively (Fig. 6a). In our laboratory, COM crystals were prepared by mixing equimolar calcium chloride (10 mM) and sodium oxalate (10 mM) solutions at a constant speed of 1 ml/min according to the method described by Pak et al. [12]. Our original COM crystals were identified as pure calcium oxalate monohydrate using an X-ray powder diffraction method.

Experiments on the effect of COM crystals on MDCK cell multiplication

Measurement of cell multiplication

MDCK cells were plated at 6×10^5 cells/well in MEM with 10% FCS as described above. Twelve hours later, the medium was aspirated and replaced with fresh MEM containing 3% FCS in which 200 or 400 μg COM crystals was suspended. Twelve hours later, living cells were dissociated with 0.25% trypsin containing 5 mM ethylenediaminetetraacetic acid (EDTA) and counted in a haemocytometer using the dye exclusion technique.

Measurement of cell viability

In the same experimental system, the cell viability was measured by MTT assay [11]. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT, Wako) was dissolved at 5 mg/ml in phosphate-buffered saline (PBS) solution. Ten hours after replacement of the medium with fresh MEM containing 200 or 400 μg COM crystals, 100 μl MTT solution was added directly to the medium and cells were incubated for 2 more hours. After removal of the medium, 1 ml isopropanol was added to each well for solubilization of the formazan crystals, and the optical density of the plates was measured on a microculture plate reader (Corona Electric, MTP-32, Ibaragi, Japan) using a test wavelength of 550 nm and a reference wavelength of 630 nm.

Experiments on cellular uptake of crystals

Morphological studies

1. Phase-contrast microscopy: MDCK cells cultured in the presence of 200 μg of COM crystals for 12 h were rinsed twice with Hank's balanced salt solution (HBSS) and observed by phase contrast microscopy as they were living in the well.
2. Light microscopy: MDCK cells cultured in the same manner were rinsed twice with HBSS and treated with 0.25% trypsin containing 5 mM EDTA. The cells were dispersed by a vigorous pipetting maneuver and living cells were observed by light microscopy without fixation.
3. Scanning electron microscopy: MDCK cells cultured in the same manner were prepared for SEM either in the monolayer or dispersed condition. The dispersed cells that were prepared by trypsinization and pipetting were transferred to a poly-L-lysine (Sigma)-coated glass piece. Both specimens in monolayer and dispersed conditions were fixed in 2.0% glutaraldehyde in 0.1 M PBS, pH 7.4, for 4 h. They were then dehydrated in a graded acetone series and dried in a critical point drying apparatus (Hitachi, HCP-2, Ibaragi, Japan). Specimens were coated with Pt-Pd in an ion sputter (Hitachi, E-101) and examined with a Hitachi S-2300 SEM at 15 kV.

Measurement of cellular uptake of crystals

At specific times after replacement of the medium with fresh MEM containing 200 μg COM crystals, the cells were dissociated with 0.25% trypsin containing 5 mM EDTA. Individual cells were observed under light microscopy, and it was assessed whether each cell

was associated with COM crystals or not. For each culture, 100 or more cells were assessed, and the total cell number and the number of cells associated with crystals were recorded.

Effect of glycosaminoglycans (GAGs) on the cellular uptake of crystals

Just before the addition of fresh medium with crystals, a monolayer of the cells was rinsed for 2 min with 0.001–1 mg/ml sodium pentosan polysulphate (SPP; Sigma), heparin (Nacalai Tesque), or chondroitin sulphate C (Nacalai Tesque), all of which were dissolved in HBSS. Six hours after the addition of fresh medium containing 200 μg COM crystals, the cells were counted as described above.

Statistics

Data were compared using Student's t-test and $P < 0.05$ was accepted as significant. Values are expressed as means ± SE.

Results

Effect of COM crystals on MDCK cell multiplication

Cell multiplication and viability

Compared with that in control cultures, the number of cells cultured in the presence of 200 and 400 μg COM crystals for 12 h was significantly increased, by 19% ($P < 0.01$) and 16% ($P < 0.05$), respectively. Assessment of the effect of the COM crystals on MDCK cell viability using the MTT assay revealed that the relative optical density of the plates in which cells were cultured in the presence of 200 and 400 μg COM crystals for 12 h was, respectively, 3% ($P < 0.05$) and 8% ($P < 0.001$) greater than that of the controls (Fig. 1).

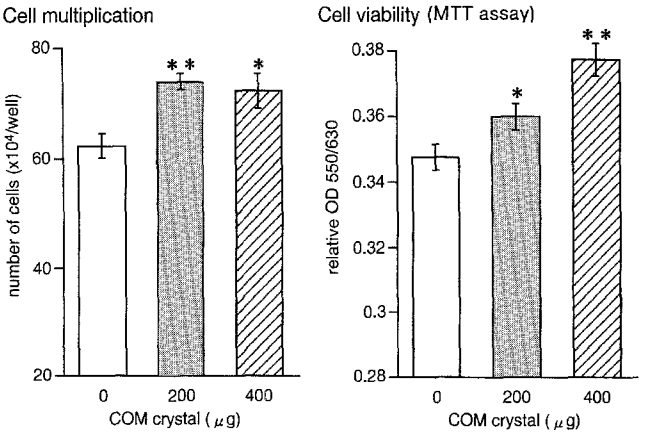

Fig. 1 Effect of COM crystals on MDCK cell multiplication and viability. Exposure of 200 μg (*shaded bars*) or 400 μg (*hatched bars*) COM crystals to MDCK cells significantly accelerated cell multiplication and activity. Each value is the mean ± SE for six cultures. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the value for control cultures (*open bars*)

Effect of crystal amount on cell multiplication

The amounts of COM crystals added to the culture medium varied from 0 to 200 μg. The multiplication of MDCK cells showed an increase proportional to the amount of COM crystals from 0 to 150 μg, above which the increase plateaued off (Fig. 2).

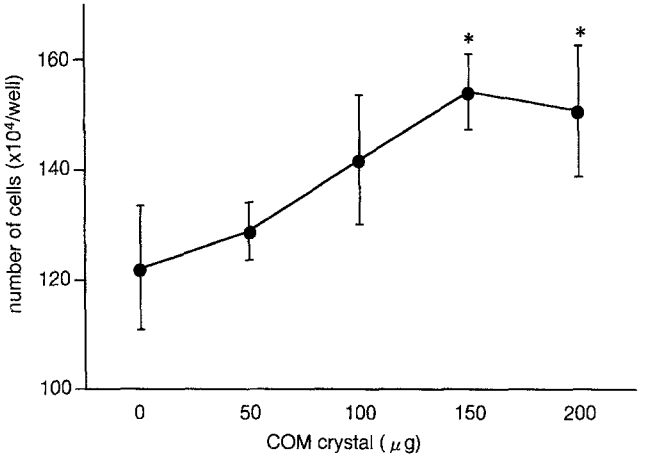

Fig. 2 Effect of crystal amount on cell multiplication. After 8×10^5 cells were cultured for 12 h, the medium was replaced with fresh MEM containing 3% FCS in which COM crystals were added in the various amounts *indicated on the abscissa*. The cell multiplication was dependent on crystal amount up to the plateau value of 150 μg. Each value is the mean ± SE for six cultures. $^{*}P < 0.05$ compared with the value for control cultures

Crystal-type specificity in the effect of cell multiplication

In order to determine whether the accelerating effect of COM crystals on MDCK cell multiplication was crystal-type specific, experiments were carried out using two other calcium-containing crystals, HA and BR. The number of cells at 12 h in cultures containing 400 μg COM crystals was significantly increased by 15.9% compared with that in controls ($P < 0.05$), while there was no difference from the control value for culture containing 400 μg HA (−0.6% compared to controls) or BR crystals (−3.5% compared to controls). The accelerating effect of crystals on cell proliferation thus showed specificity for crystal type.

Cellular uptake of crystals

Microscopic findings

Figure 3a is a phase-contrast microscopic picture of MDCK cells cultured in the medium with 200 μg COM

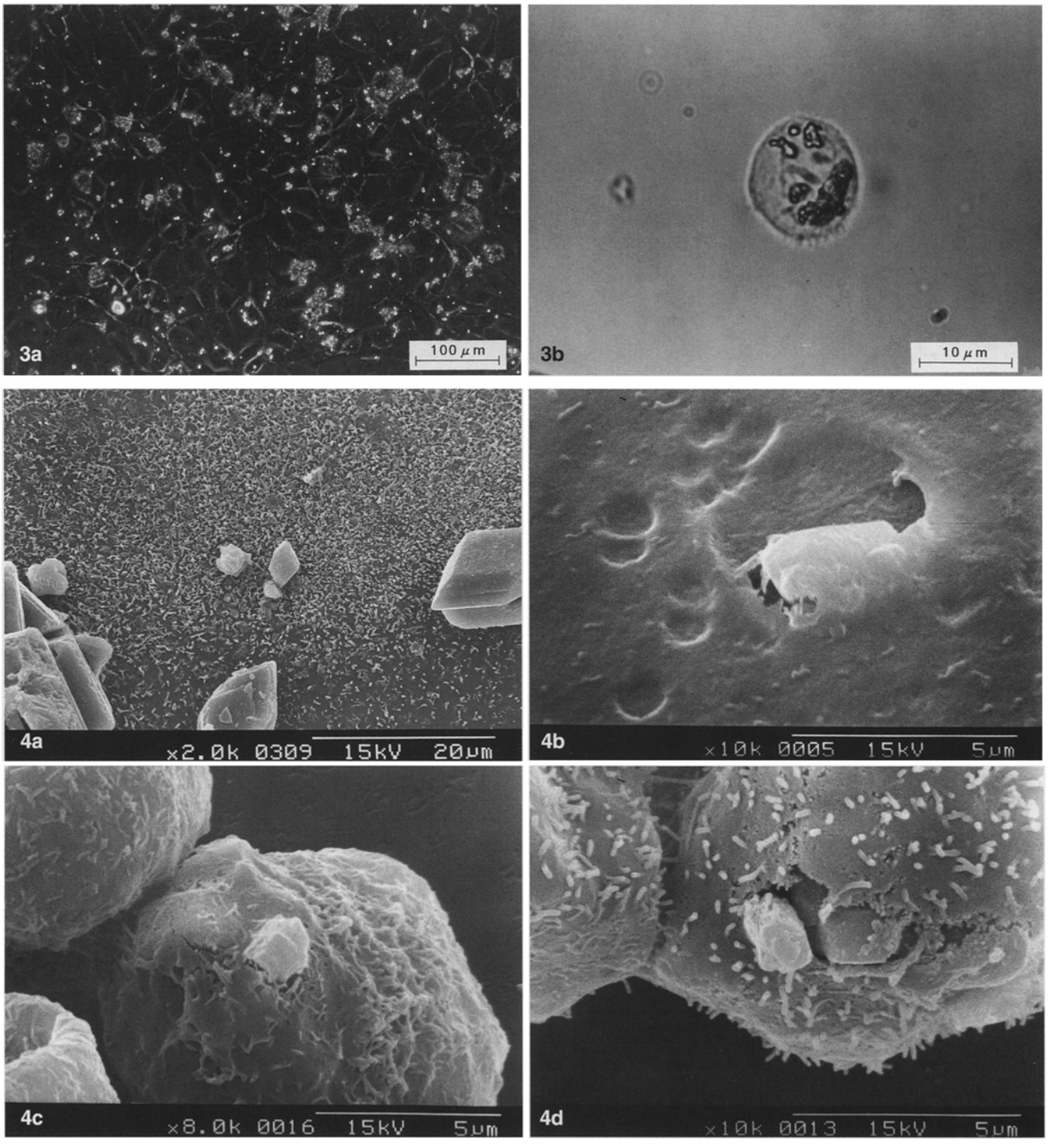

Fig. 3 **a** Phase contrast photomicrograph of MDCK cells (original magnification × 200). The COM crystals adhered predominantly to the cell surface rather than to the intercellular space. **b** Light photomicrograph of MDCK cells (original magnification × 400). The COM crystals were not dissociated from the cells in spite of trypsinization, which we call crystal-associated cells

Fig. 4 SEM image of MDCK cells. **a**, **b** Monolayer of MDCK cells. COM crystals appeared not only to lie on the cell surface but also to be covered with the cell membrane. **c**, **d** Trypsinized MDCK cells. Some crystals appeared to be located on the cell surface (adhesion) and others within the cell (endocytosis)

crystals for 12 h and rinsed twice with HBSS. COM crystals predominantly adhered to the cell surface rather than the intercellular space. In Fig. 3b, even when MDCK cells cultured in the same manner were treated with 0.25% trypsin containing 5 mM EDTA, some of the COM crystals were not dissociated from the cells. It was assumed that the crystals adhered to the cell surface or were internalized by the cells by some biological mechanisms.

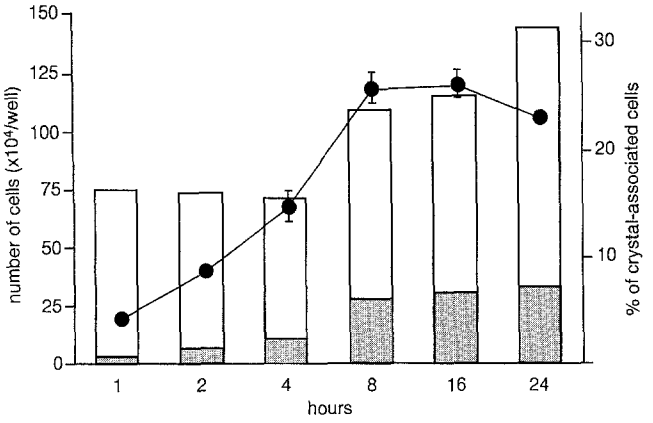

Fig. 5 Time course in cellular uptake of crystals. *Shaded* and *open bars* show the numbers of cells with and without crystals, respectively, the scale of which is indicated *on the left y-axis. Solid circles* show the percentage of crystal-associated cells to total cells, the scale of which is *indicated on the right y-axis.* Number and percentage of crystal-associated cells increased in a time-dependent manner. Each value is the mean ± SE for six cultures

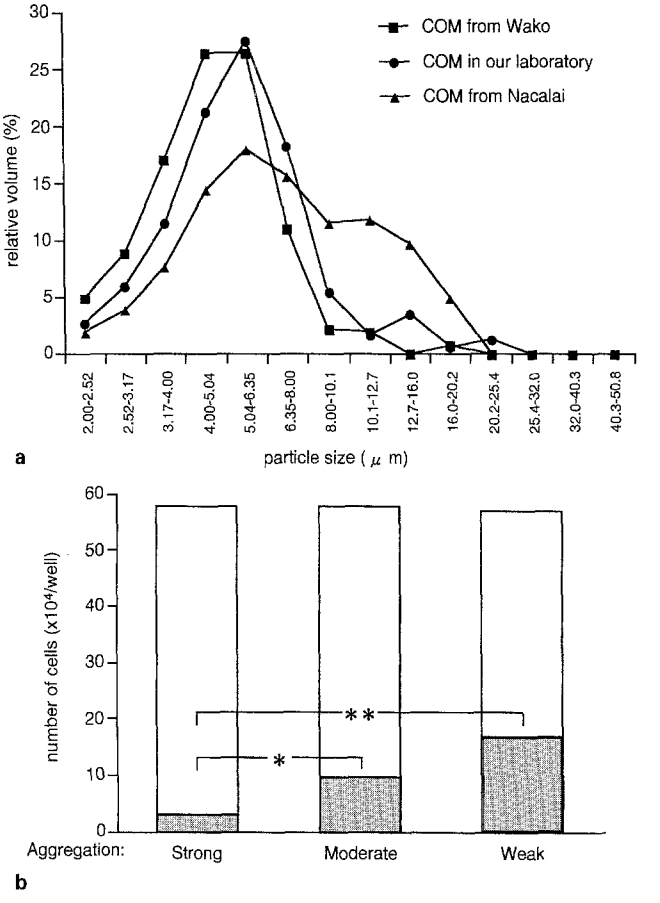

Fig. 6 a Particle volume distribution curve of COM crystals from three sources, which were measured with a TAII Coulter counter. These crystals differed in their degrees of aggregation even after the treatment of ultrasound, with those from Nacalai Tesque, manufactured in our laboratory, and from Wako showing strong, moderate, and weak aggregation, respectively. **b** Effect of crystal aggregation on cellular uptake. *Shaded bars* and *open bars* indicate the number of cells associated with crystals and not associated crystals, respectively. The cellular uptake of crystals with strong aggregation was significantly less than that of crystals with moderate or weak aggregation ($*P < 0.01$ and $**P < 0.001$, respectively). Each value is the mean for six cultures

SEM findings

Figure 4a, b shows SEM pictures of the monolayer of MDCK cells cultured in the presence of 200 μg COM crystals for 12 h. Some crystals appeared not only to lie on the cell surface but also to be covered with a plasma membrane. In the trypsinized cells, some crystals appeared to be located on the cell surface and others within the cell (Fig. 4c, d). The former were thought to "adhere" to the plasma membrane and the latter to "be endocytosed" by the cell.

Time course in cellular uptake of crystals

When the MDCK cells were cultured in the presence of 200 μg COM crystals, the numbers of both total cells and those associated with crystals increased in a time-dependent manner, and the percentage of crystal-associated cells to total cells was also increased in a time-dependent manner. This percentage was 4.4% at 1 h and had increased to 26% by 8 h, plateauing thereafter (Fig. 5).

Crystal-type specificity in cellular uptake of crystals

To determine whether the adhesion to the cell surface and endocytosis were crystal-type specific, we carried out experiments using the two other calcium-containing crystals, HA and BR. The percentage of crystal-associated cells was 56.2% for cells cultured in the presence of 1 g COM crystals for 6 h, but only 4.1% and 0%, respectively, for those cultured in the presence of 1 g HA and BR crystals. The cellular uptake of crystals showed specificity for crystal type.

Effect of crystal aggregation on cellular uptake of crystals

COM crystals from three sources which differ in their degrees of aggregation were added in culture medium at a final dose of 200 μg (Fig. 6a). Cells cultured in the presence of the crystals with strong aggregation showed a significantly lower percentage of crystal-associated cells at 6 h than those cultured in the presence of the crystals with either moderate or weak aggregation ($P < 0.01$ and $P < 0.001$) (Fig. 6b).

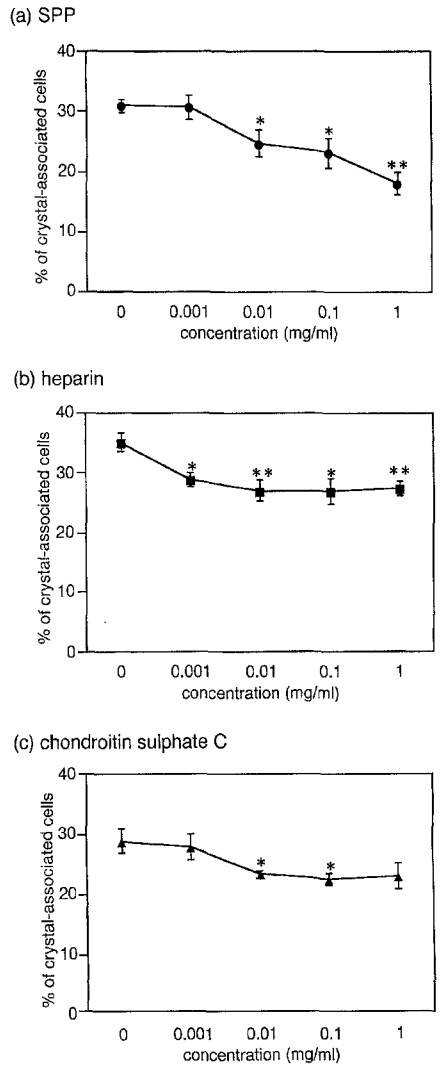

Fig. 7 Inhibitory activities of GAGs on cellular uptake of crystals. The pre-treatment with **a** SPP, **b** heparin, and **c** chondroitin sulphate C produced a significant and concentration-dependent reduction of the cellular uptake of crystals. The inhibitory activity was the strongest for SPP, followed by chondroitin sulphate C and heparin, respectively. Each value is the mean for six samples. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with control values

Inhibitory activities of GAG on cellular uptake of crystals

The cellular uptake of crystals showed significant and concentration-dependent inhibition following the pre-treatment of the cells with each of the three GAGs. The inhibitory activity was the strongest for SPP, followed by chondroitin sulphate C and heparin, respectively (Fig. 7).

Discussion

Evidence regarding urinary flow rates, anatomical dimensions, and urinary supersaturation indicates that the retention of crystals within the tubular lumen is a necessary condition for the formation of clinical kidney stones [2]. However, these earliest events in the formation of the kidney stones have not been well understood.

In this study, we investigated the interactions between cultures of MDCK cells and calcium-containing crystals using methods developed at our institution. We noticed that all results in this study were obtained in the cultures of MDCK cells that were nearly confluent. We adapted these conditions in order to maintain the mitogenic activity of the cells and confirmed that most of the cells used for the study contacted each other and showed no morphological differences from the cells in completely confluent cultures.

COM crystals, the most common constituents of kidney stones, were endocytosed by MDCK cells in culture and accelerated cell multiplication. MTT assay revealed that MDCK cells cultured with COM crystals demonstrated a higher activity than control cells. It is suggested that the attachment of crystals to the plasma membrane and endocytosis of crystals might be related to the cell mitosis. Lieske et al. [9] recently noted that the COM crystal is readily internalized, initiating DNA synthesis and stimulating cell multiplication, in cultures of monkey kidney epithelial cells (BSC-1 line) and MDCK cells. They speculated that the dissolution of the internalized crystal and elevation of the intracellular calcium concentration advanced the G1 phase of the cell cycle, leading to DNA synthesis.

Light and scanning electron microscopic observation of MDCK cells cultured in the presence of COM crystals revealed that some crystals adhered to the plasma membrane and others were internalized by the cell. Some biological mechanisms might be related to these phenomena, since neither treatment with trypsin containing EDTA nor the pipetting maneuver dissociated the crystals from MDCK cells.

The cellular uptake of COM crystals and crystal-induced cell multiplication were found to be crystal-type specific; COM crystals induced these cellular responses, while HA and BR crystals did not. Furthermore, COM crystals with strong aggregation were not as frequently endocytosed by MDCK cells as those with weak aggregation. These results suggest that the size or structure of crystals might be closely related to the induction of these cellular responses. However, further study is required to clarify the essential mechanisms of these crystal-cell interactions.

It is well known that disturbance of the GAG layer of the urothelium, especially in the urinary bladder, is associated with an increased number of adherent crystals, while treatment with exogenous GAGs significantly reduces the crystal adherence [5, 13, 14]. We observed that GAGs significantly reduced the cellular uptake of crystals. Although the mechanism of the phenomenon requires further study, we speculate that GAGs might possibly reduce the cell capacity to adhere

to and internalize the crystals, and urinary GAGs might play some critical roles in preventing crystal adhesion and endocytosis in the cortical tubular cells as well as the urinary bladder cells. Recently, Lieske et al. [7] noted that diverse substances including heparin inhibited the endocytosis of COM crystals by acting on the cell surface, rather than on the crystal.

Recently, we reported that COM crystals adhered to the intact MDCK cells in a time- and concentration-dependent manner, and that this adherence might be one of the earliest processes in the formation of kidney stones [1]. Crystal-induced cell multiplication may cause a disturbance of the tubular epithelium, where the flow rate of fluid is reduced and intraluminal crystals are exposed to epithelium for a period long enough for attachment to occur. On the other hand, engulfment of crystals and active cell division might cause structural change of the plasma membrane or cytoskeleton, which is noted to enhance the cellular capacity for crystal binding or endocytosis [7, 17].Thus, a crystal-induced cellular response might provide a site for crystal adhesion to the epithelium and anchored crystal aggregation and growth.

We assumed that these crystal-cell interactions, that is, cellular uptake of crystals and cell proliferation resulting from this uptake, might be necessary for crystal aggregation in the urine flow of the renal tubules. However, the essential mechanism and severity of these phenomena in stone pathogenesis are still unknown and require further investigation.

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