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ADHESION AND ENDOCYTOSIS OF CALCIUM OXALATE CRYSTALS ON RENAL TUBULAR CELLS

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Abstract

The present investigation was designed to study interactions between Madin-Darby canine kidney (MDCK) cells and calcium oxalate monohydrate (COM) crystals and to clarify the significance of these crystal-cell interactions in stone pathogenesis.

MDCK cells cultured in the presence of COM crystals showed a time-dependent uptake of crystals; this was specific for COM crystals. In the dynamic model system designed to study these phenomena under more physiological conditions, COM crystals adhered to the cell surface and were subsequently internalized. In this endocytotic process, the microvilli of the cell appeared to play an important role. The observation by scanning electron microscopy of complexes consisting of aggregated COM crystals and cell debris led us to speculate that adhesion and endocytosis of crystals might provide the calculus nidus for aggregation and retention of crystals in the renal tubule. Furthermore, glycosaminoglycans and the macromolecular fraction of human urine were shown to have the ability to inhibit the cellular uptake of crystals.

Evidence that similar processes may also occur *in vivo* was obtained using an experimental stone model in rats. Our experiments revealed that most of the COM crystals adhered to the tubular cells and some crystals were endocytosed by the cell. Thus, these crystal-cell interactions might be one of the earliest processes in the formation of kidney stones. Further elucidation of the mechanism and the regulatory factors involved in this process may provide new insight into stone pathogenesis.

Key Words: Crystal-cell interaction, Madin-Darby canine kidney (MDCK) cells, calcium oxalate crystal, adhesion, endocytosis, microvillus.

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Introduction

Considering urinary flow rates, anatomical dimensions and urinary supersaturation, the retention of crystals within the tubular lumen is likely to be a necessary condition for the formation of kidney stones [2]. However, the mechanisms by which crystals are trapped in the tubular lumen are poorly understood.

The relation between crystals and renal epithelial cells has been noted for a long time; for example, the existence of papillary casts and Randall's plaque hypothesis [19] have been known as potential causative factors in the pathogenesis of human kidney stones. While there is circumstantial clinical evidence to suggest that human renal epithelial cells react with urinary crystals [11, 15, 16], Khan *et al.* [8] observed crystal attachment to the brush border of proximal tubules in experimental rats. Furthermore, recent advances in cell biology techniques have led to active investigations of crystal-cell interactions *in vitro* systems. Mandel, along with other researchers, reported the binding of calcium oxalate monohydrate (COM) crystals to rat renal papillary collecting tubular cells in primary culture [14, 20, 21, 23]. On the other hand, Lieske *et al.* [12] noted that COM crystals were endocytosed by cultured renal epithelial cells and induced cell proliferation. 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. As a model system to study these interactions, we employed COM crystals and cultures of Madin-Darby canine kidney (MDCK) cells. MDCK cells exhibit many characteristics of the cortical collecting tubular cells of the kidney [3, 22]. We have already reported that COM crystals adhered to the intact surface of MDCK cells in a time- and concentration-dependent manner, and that cell injuries induced by 0.1 M HCl or gentamicin and the presence of glycosaminoglycans reduced crystal attachment [1].

In the present study, we carried out three types of

experiments to clarify some of the mechanisms of crystal retention in the renal tubules: (1) We examined the cellular uptake of COM crystals and the effects of glycosaminoglycans (GAGs) in the "static model system." (2) We developed the "dynamic model system" to observe these crystal-cell interactions under more physiological conditions. Using this system, we attempted to examine the specific issues of the fate of COM crystals once adhered to the renal tubular cells and the effect of human urine on these interactions. (3) We also studied the kidney in a rat model of nephrolithiasis to evaluate the significance of these phenomena in stone pathogenesis.

Materials and Methods

Cell culture

MDCK cells were obtained from the Laboratory Products Div., Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and subcultured in minimum essential medium (MEM; Gibco, Grand Island, NY) 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% CO₂ and 95% air atmosphere. In the static model system, 6 x 10⁵ cells were cultured in 6-well culture plates (Nippon Becton Dickinson, Tokyo, Japan) for 12 hours, and the subconfluent cultures were used for study. In the dynamic model system, 6 x 10⁴ cells were seeded onto 26 mm x 76 mm polystyrene culture plates and the subconfluent cultures were used for study two days later. Under the phase-contrast microscopy and scanning electron microscopy (SEM), most of the cells cultured in this manner contacted to 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 treated with ultrasound for 15 minutes in order to obtain uniform crystal conditions. Scanning electron micrographs verified the average sizes of these crystals {with COM, HA and BR showing 0.7 μm (0.4-2.0 μm), 1.8 μm (0.8-6.5 μm) and 3.3 μm (1.2-6.9 μm), respectively}, and showed that the treatment with ultrasound removed the aggregation of these crystals and did not alter the size and structure of the individual crystals.

"Static model system" of crystal-cell interactions

MDCK cells were plated at 6 x 10⁵ cells per 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 μg of COM

crystals were suspended.

Measurement of cellular uptake of crystals At various times after addition of COM crystals, cells were rinsed three times with Hank's balanced salt solution (HBSS) in order to remove the non-adherent crystals, detached with 0.25% trypsin containing 5 mM ethylenediaminetetraacetic acid (EDTA) and dispersed by pipetting maneuver. Individual cells were observed under light microscopy to assess whether they were associated with COM crystals or not. For each culture, one hundred or more cells were assessed, and the total cell number and the number of cells associated with crystals were counted using a hemocytometer. As a preliminary study, the same volume of 0.4% trypan blue and dispersed cell suspension were mixed and counted on a hemocytometer, which revealed that more than 95% of the MDCK cells were still viable after they had been incubated in the presence of COM crystals for 24 hours.

Effect of GAGs on cellular uptake of crystals Just before the addition of crystals, a monolayer of the cells was exposed to GAG solution for 2 minutes. After removal of GAG solution by aspiration, fresh medium containing 200 μg of COM crystals was added to each well. Six hours later, the cells were assessed as described above. GAG solutions examined in this study were 0.001 to 1 mg/ml of sodium pentosan polysulphate (SPP; Sigma Chemical Co., St. Louis, MO), heparin (Nacalai Tesque, Kyoto, Japan), or chondroitin sulphate C (Nacalai Tesque), all of which were dissolved in 2 ml of HBSS. The inhibitory activities of the each of GAGs were calculated, as compared to the control cultures which were exposed to a 2 minute pre-treatment with HBSS without GAGs.

Preparation for scanning electron microscopy MDCK cells cultured in the presence of 200 μg COM crystals for 12 hours were rinsed three times with HBSS and trypsinized. The cells were transferred to a poly-L-lysine (Sigma) coated glass piece. The glass pieces with attached cells were fixed in 2.0% glutaraldehyde in 0.1 M phosphate-buffer, pH 7.4, for 4 hours. They were then dehydrated in a graded acetone series and dried in a critical point drying apparatus (HCP-2, Hitachi, Ibaragi, Japan). Specimens were coated with a Pt-Pd in an ion sputter (E-101, Hitachi) and observed with a Hitachi S-2300 scanning electron microscope.

"Dynamic model system" of crystal-cell interactions

We developed the "dynamic model system" in order to examine crystal-cell interactions in more physiological state. Subconfluent cultures of MDCK cells on polystyrene plates were rapidly rinsed with HBSS. COM crystals were suspended in Tris-HCl buffer containing 0.15 M NaCl (pH 7.4) at a final concentration of 2 mg/ml, which was constantly stirred at 200 rpm during

the experiment. The culture plate was then dipped vertically into a stirred COM crystal suspension and held there for 5 minutes.

This experimental system has two features mimicking those of the renal physiology: first, the monolayer of the cells is exposed to crystals in a vertical position without the possibility of spontaneous contact of the crystals due to gravity; second, stirring of the crystal suspension produces a dynamic exposure of the crystals to the cells, as in the renal tubules.

SEM observations of the cellular uptake of crystals After removal of non-adherent crystals by washing the plate three times with HBSS, the cells were subsequently re-cultured in medium without crystals, with the plate placed in vertical position. At specified times thereafter, the culture plate was rinsed three times with HBSS, and the cells on the plate were prepared for SEM without trypsinization. Fixation and the latter procedure for SEM were as described above. A preliminary study, using trypan blue exclusion technique, revealed that more than 95% of the MDCK cells were still viable after they were exposed to COM crystals and cultured for 30 hours in vertical position.

Effect of the human urine on cellular uptake of crystals (1) Urine samples: The 24-hour urine samples were collected from two healthy volunteers. For the experiment, each urine sample was centrifuged at 1,500 rpm for 5 minutes and the supernate was provided as the whole urine sample. Ten and 100-fold dilutions of whole urine samples were made with Tris-HCl buffer containing 0.15 M NaCl, and also used for study. While, the urinary macromolecules (molecular weight > 5,000 D) was extracted from whole urine by gel-filtration using Sephadex G-25 medium (Pharmacia Biotech, Uppsala, Sweden), and then diluted with Tris-HCl buffer to the original volume, which was provided as a macromolecular fraction. The macromolecular fraction should contain same quantity of urinary macromolecules as the original whole urine. COM crystals were suspended in these urine samples at a final concentration of 2 mg/ml. (2) Measurement of cellular uptake of COM crystals: MDCK cells on the polystyrene plate were exposed to COM crystals in urine samples for 5 minutes using the dynamic model system. After removal of non-adherent crystals by washing the cell surface three times with HBSS, the adherent COM crystals and the cells on the plate were lysed with 5 ml of hydrochloric acid (6 N) and harvested using a cell scraper. The harvested solutions were allowed to stand for more than 1 hour and were then centrifuged at 1,500 rpm for 10 minutes. The quantitative analysis of the adherent COM crystals was conducted by measuring the calcium concentration of the supernatants using atomic absorption spectroscopy. The

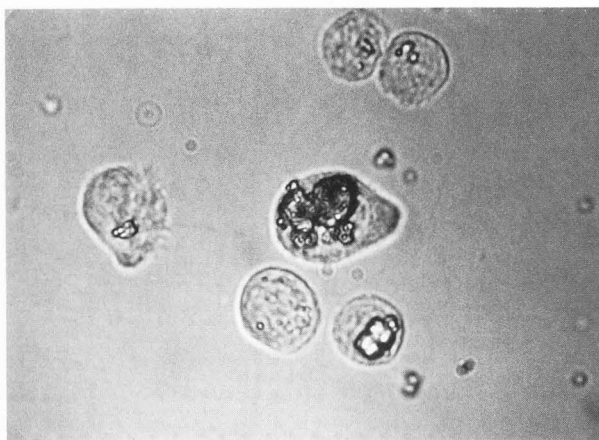


Figure 1. Light micrograph of MDCK cells cultured in the presence of 200 µg of COM crystals for 12 hours. COM crystals still adhered to some of the cells even after the trypsinization and vigorous pipetting maneuver. Photo width (P.W.) = 60 µm.

exact amount of adherent crystals was calculated by subtracting the calcium content of control cells from the total calcium content derived from cells exposed to crystals. The inhibitory activity of each urine sample to suppress the cellular uptake of COM crystals was calculated by the following formula:

$$\text{Inhibitory activity} = \{100 (a-b) / a\}$$

where a = adherent COM crystals (Ca concentration, ppm) in Tris-HCl buffer; and b = adherent COM crystals (Ca concentration, ppm) in the urine samples.

Histological study of the kidney in rat model of nephrolithiasis

The experiments were carried out with male Sprague-Dawley rats (Japan Clea Inc., Osaka, Japan) each weighing 80-110 g. A high calcium diet was prepared by the addition of 112 mg of calcium gluconate monohydrate to 1 g of standard chow (Oriental Yeast Co., Ltd., Tokyo, Japan). Three rats were fed high calcium diet *ad libitum*, were allowed to drink distilled water for one week, and then were injected intraperitoneally with 10 mg of sodium oxalate per 100 mg of body weight, according to the method described by Khan *et al.* [8]. Two hours later, the rats were killed, and the kidneys were examined with polarizing light optical microscopy.

Statistics

Data were compared using Student's t-test, and $p < 0.05$ was accepted as significant. Values are expressed as means \pm standard error (SE).

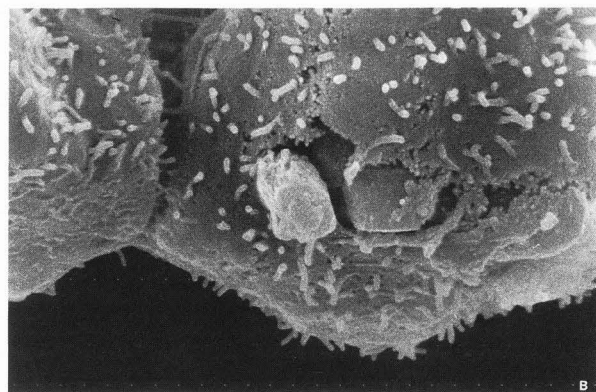
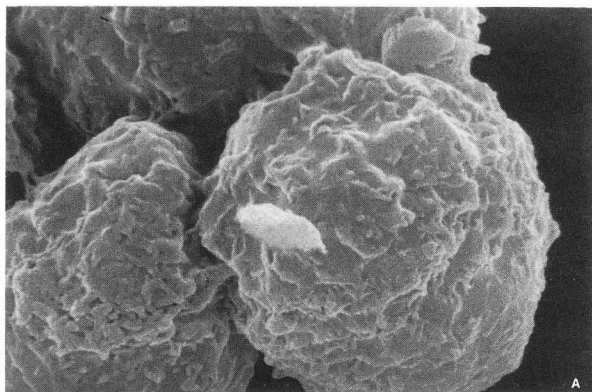


Figure 2. Scanning electron micrographs of MDCK cells cultured in the same manner as Figure 1. (A) COM crystal adheres to the cell surface. P.W. = 14 μm . (B) COM crystals are internalized into the cell. P.W. = 11 μm .

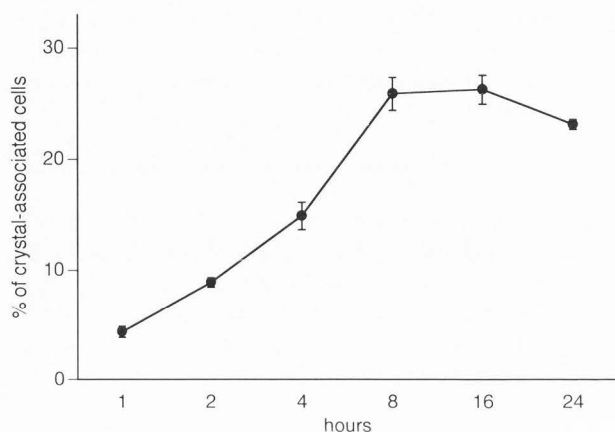


Figure 3. Time course in cellular uptake of crystals. When the MDCK cells were cultured in the presence of 200 μg of COM crystals, the percentage of crystal-associated cells increased in a time-dependent manner with plateauing. Each value is the mean \pm SE for 6 cultures.

Results

Crystal-cell interactions in the "static model system"

Microscopic findings MDCK cells cultured in the presence of 200 μg of COM crystals for 12 hours were examined. Light microscopy showed that COM crystals were still binding to some of the cells even after the trypsinization and vigorous pipetting maneuver (Fig. 1). SEM observations revealed that some of these crystals appeared to be located on the cell surface (Fig. 2A) and others within the cell (Fig. 2B). The former were thought to "adhere" to the plasma membrane and the latter to "the endocytosed" by the cell.

Time course in cellular uptake of crystals When the MDCK cells were cultured in the presence of 200 μg

of COM crystals, the percentage of crystal-associated cells increased in a time-dependent manner. This percentage was 4.4% at 1 hour and increased to 26% by 8 hours, plateauing thereafter (Fig. 3).

Crystal-type specificity in cellular uptake of crystals To determine whether the cellular uptake of crystals was crystal-type specific, experiments using two other calcium-containing crystals, HA and BR, were carried out. The measurements of crystal-associated cells were performed on trypsinized cells. While 56.2% of the cells exposed to 1 mg COM crystals for 6 hours were associated with crystals, the percentage of crystal-associated cells following incubation with 1 mg HA or BR crystals was only 4.1% and 0%, respectively (Fig. 4). Thus, cellular association of crystals showed specificity for crystal type.

Inhibitory activities of GAGs on cellular uptake of crystals Figure 5 shows the inhibitory activities of each of the three GAGs on cellular uptake of crystals, when the cells were rinsed for 2 minutes with GAGs and then incubated with 200 μg of COM crystals for 6 hours. The cellular uptake of crystals showed significant and concentration-dependent inhibition following the pre-treatment with each of the three GAGs. The inhibitory activity was strongest for SPP, followed by chondroitin sulphate C and heparin, respectively.

Crystal-cell interactions in the "dynamic model system"

SEM findings of the cellular uptake of crystals COM crystals adhered to the cell surface immediately after exposure and rinsing with HBSS. Most of the crystals appeared to merely lie on the plasma membrane (Fig. 6A). However, in the high-magnification view of some crystals, many microvilli of the cell were already elongated toward the crystals (Fig. 6B). Thus, the adhesion of COM crystals to the MDCK cells could occur

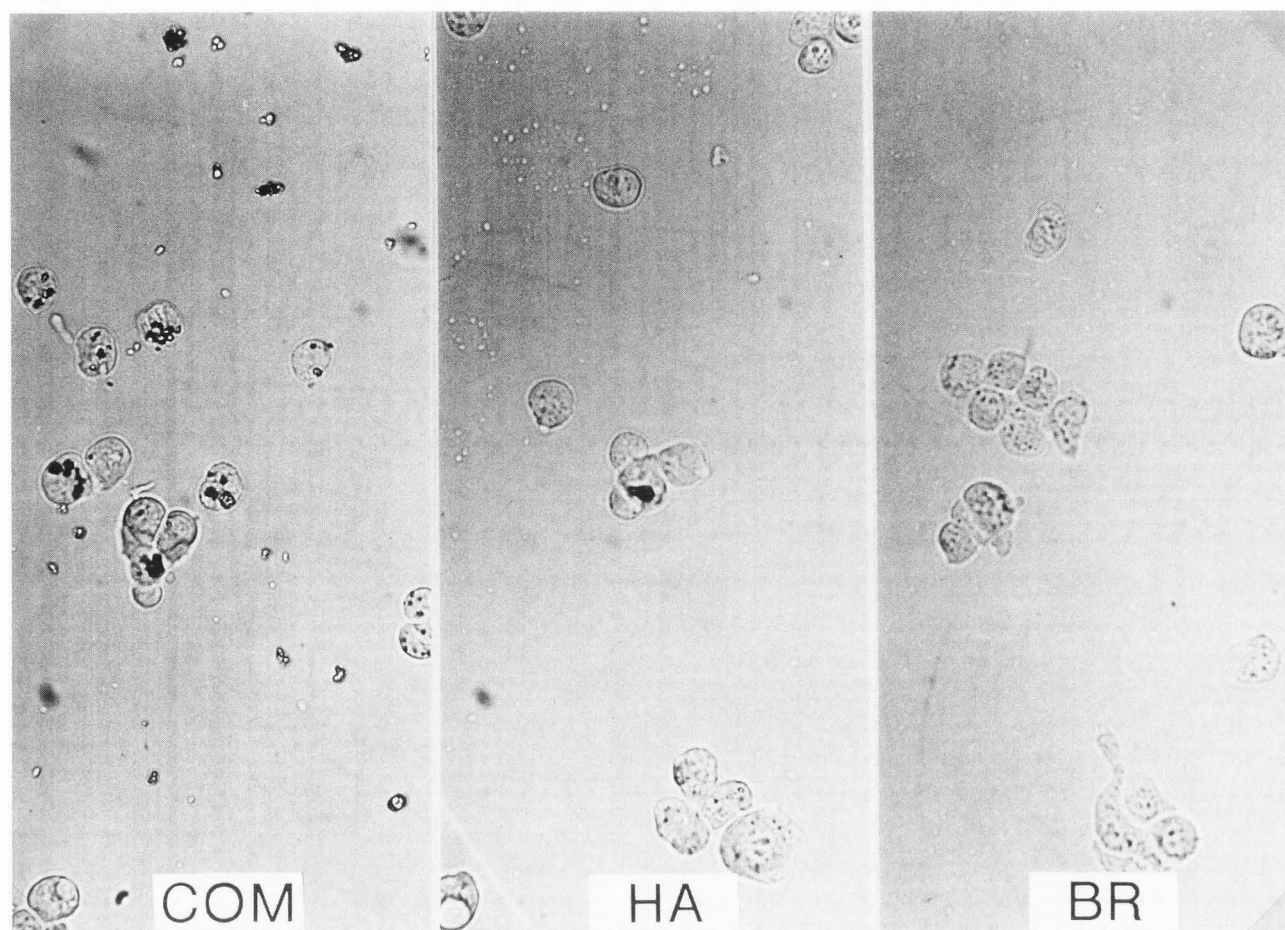


Figure 4. Light micrograph of MDCK cells cultured in the presence of 1 mg of COM (A), HA (B) and BR (C) crystals for 6 hours. Cells had been treated with 0.25% trypsin containing 5 mM EDTA. The cellular uptake were specific for COM crystals. P.W. (for each panel) = 100 μ m.

even in the dynamic condition. At one hour after the exposure of COM crystals, microvillar projections had covered the crystal surface (Fig. 6C). Moreover, some crystals appeared to be surrounded by the plasma membrane and to be nearly internalized into the cell (Fig. 6D). At 6 hours, there was some protrusion of degenerated plasma membrane, which suggested that the COM crystal was completely covered with plasma membrane or internalized (Fig. 6E). At later times, some aggregated crystals that appeared to be a complex of cell debris and COM crystals were seen (Fig. 6F).

Effect of the human urine on cellular uptake of crystals To assess the effect of human urine on the adhesion of crystals, MDCK cells in the "dynamic model system" were exposed to COM crystals in the urine samples for 5 minutes. Compared with controls in which crystals were suspended in Tris-HCl buffer, crystal adherence in the presence of urine samples was sig-

nificantly reduced ($p < 0.01$). Dilution of the urine samples resulted in lowered capacity to inhibit crystal adherence (Fig. 7). Furthermore, we compared the inhibitory activities between the whole urine and the macromolecular fraction, either of which contains same quantity of urinary macromolecules. The inhibitory activities of the macromolecular fraction and the whole urine were $61.8 \pm 2.5\%$ and $66.9 \pm 0.6\%$, respectively in urine sample 1, and $63.5 \pm 3.0\%$ and $66.5 \pm 2.2\%$, respectively in urine sample 2, which showed no significant differences.

Histological study of the kidney in rat model of nephrolithiasis

Renal calcification in rats were induced by high calcium diet for 1 week and intraperitoneal injection of sodium oxalate (10 mg per 100 g of body weight). Examination of the paraffin sections of the rat kidneys revealed that all three rats formed COM crystals which

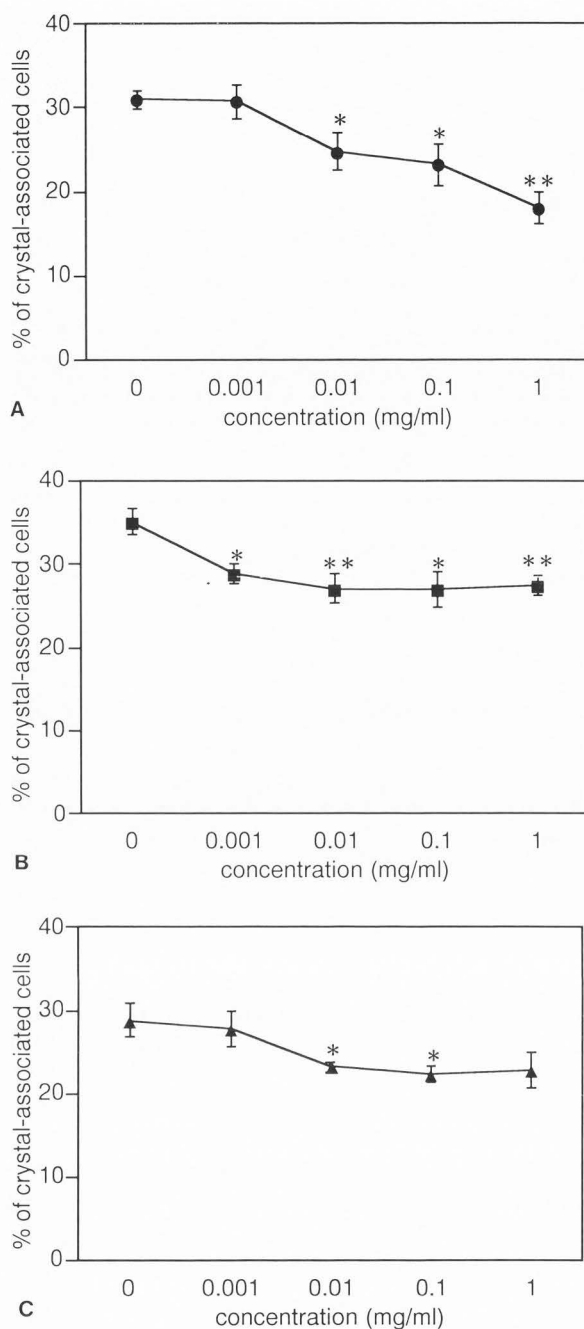


Figure 5. Inhibitory activities of GAGs on cellular uptake of crystals. A monolayer of the cells was rinsed for 2 minutes with each of the three GAGs, and then incubated with 200 μ g of COM crystals for 6 hours. The pre-treatment with (A) SPP, (B) heparin and (C) chondroitin sulphate C produced significant 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 \pm SE for 6 cultures. ** $p < 0.01$ and * $p < 0.05$ compared with the value for non-treatment controls.

were intensely birefringent, polycrystalline, and arranged in rosette or sheaf patterns with polarized light. Crystals were located both in the cortex and in the medulla, and most of them were adherent to the epithelial cells rather than floating in the tubular lumen (Fig. 8A). Moreover, a few crystals appeared to be present within the epithelial cells, and to be endocytosed by the cell (Fig. 8B).

Discussion

In the "static model system," MDCK cells cultured in the presence of COM crystals showed a time-dependent uptake of crystals. The cellular uptake of COM crystals was found to be crystal-type specific; COM crystals induced these cellular responses, while HA and BR crystals did not. It is suggested that the size or structure of crystals might be closely related to the induction of these cellular responses.

Experiments in the "dynamic model system" clarified the process of the cellular association of crystals in more physiological situation. COM crystals adhered to the cell surface and were subsequently internalized by the cells, even in the dynamic condition. Interestingly, the microvilli appeared to play an important role in the adhesion and endocytosis of COM crystals. Recently, Lieske *et al.* [13], using cultures of BSC-1 cells, noted that microvilli and actin filaments contained in its core participated in these processes. Lieske and Toback [10] also indicated that a low-K environment promoted cellular uptake of crystals by increasing the number of microvilli. The mechanisms of cellular uptake of crystals that is specific to COM have not yet been elucidated completely, although the results of some studies have suggested that specific receptors binding the COM crystals were located on the cell surface [10, 14].

On the other hand, endocytosis of COM crystals has been reported to initiate DNA synthesis and stimulate cell multiplication [5, 12], as we have also noted in a previous study using MDCK cells [9]. Crystal-induced cellular proliferation might expose new domains on the plasma membrane or cause the disturbance of the tubular epithelium, which served as a nidus for further crystal binding and anchored crystal aggregation and growth in the supersaturated urine. In addition, SEM findings revealing complexes which consisted of aggregated COM crystals and cell debris (Fig. 6F) suggested that the cells might be released from tubular epithelium with associated COM crystals. These crystal-cell complexes might occlude the tubules and cause further crystal retention. Hackett *et al.* [6] also observed crystal-cell complexes released from culture, but they attributed this to cell toxicity of COM crystals.

It is well known that the disturbance of the GAG layer of the urothelium, especially in the urinary bladder, is associated with an increased number of adherent

Calcium oxalate crystal - cell interactions

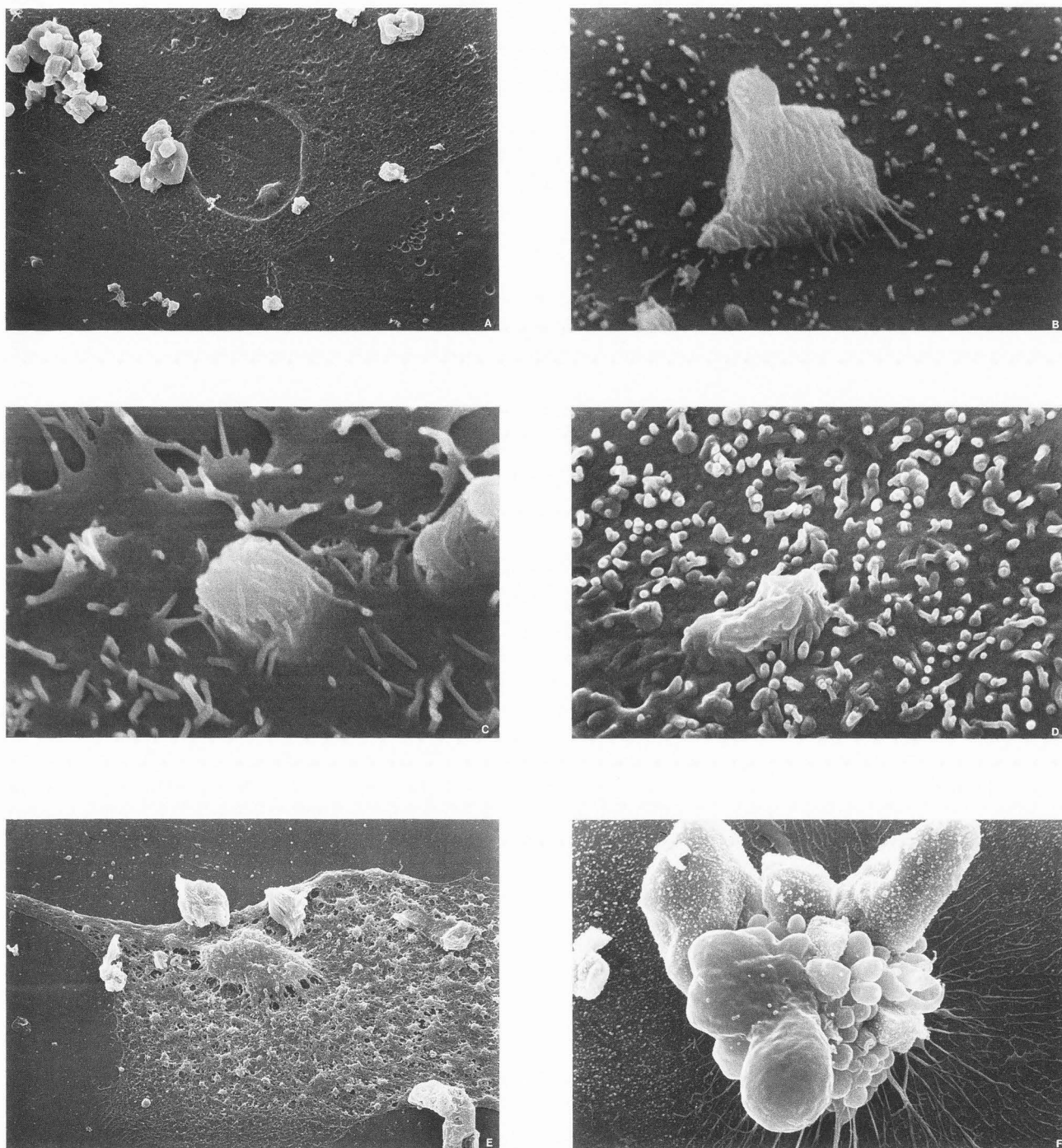


Figure 6. Scanning electron micrographs of MDCK cells exposed to 2 mg/ml of COM crystal suspension in the "dynamic model system." (A) and (B) Immediately after the crystal-exposure and rinsing with HBSS, some crystals merely lie on the plasma membrane and others are entrapped by the microvilli. (C) and (D) At 1 hour, the crystals were covered with microvillar projections and appeared to be nearly internalized into the cell. (E) At 6 hours, protrusion of degenerated plasma membrane is observed, suggesting that the COM crystal has been completely covered with plasma membrane or internalized. (F) At 24 hours, a complex that consists of aggregated COM crystals and cell debris is seen.

Photo widths (P.W.) = 75 μm (A); 11 μm (B and D); 7.5 μm (C); 57 μm (E); and 28 μm (F).

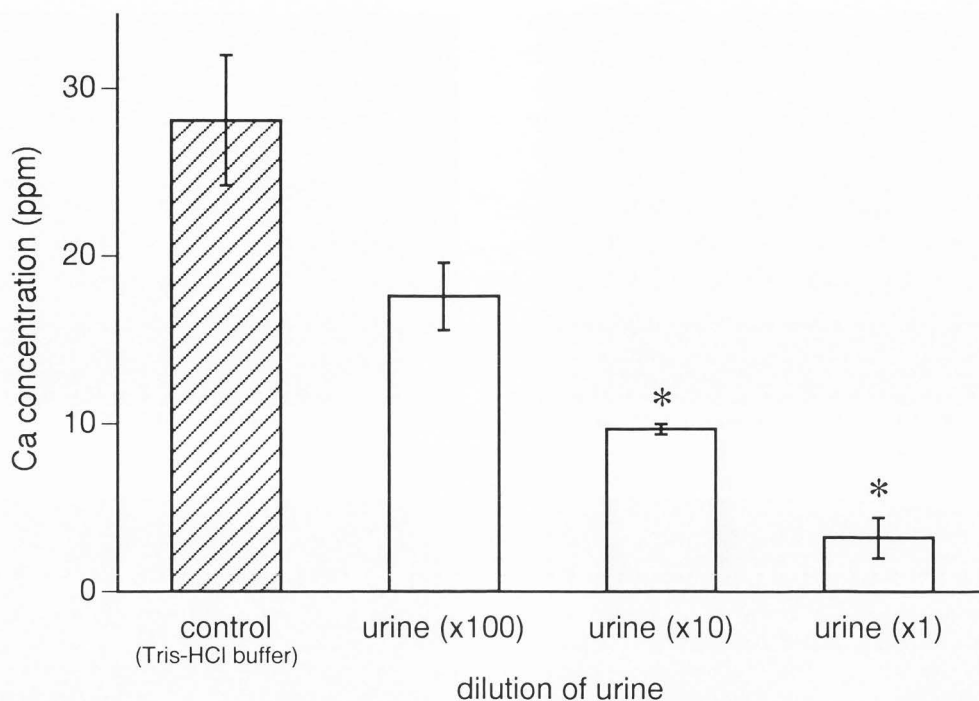


Figure 7. Effect of human urine on cellular uptake of crystals. The urine sample from a healthy volunteer was centrifuged at 1,500 rpm for 5 minutes, and the supernate and its dilutions (10 and 100-fold) were provided as whole urine. COM crystals suspended in these urine samples were exposed to MDCK cells for 5 minutes using the "dynamic model system." Human urine significantly reduced the adhesion of COM crystals, which showed the dependency on the degree of dilution. Each value is the mean \pm SE for 5 cultures. * $p < 0.01$ compared with the value for controls (Tris-HCl buffer).

Figure 8 (on the facing page). Polarized light micrographs of the kidney in rat model. Renal calcification were induced by high calcium diet for 1 week and intraperitoneal injection of sodium oxalate (10 mg per 100 g of body weight). (A) Most COM crystals are adherent to the tubular epithelium rather than floating in the tubular lumen. P.W. = 900 μ m. (B) COM crystal (arrow) appears to be endocytosed by the cell. P.W. = 900 μ m.

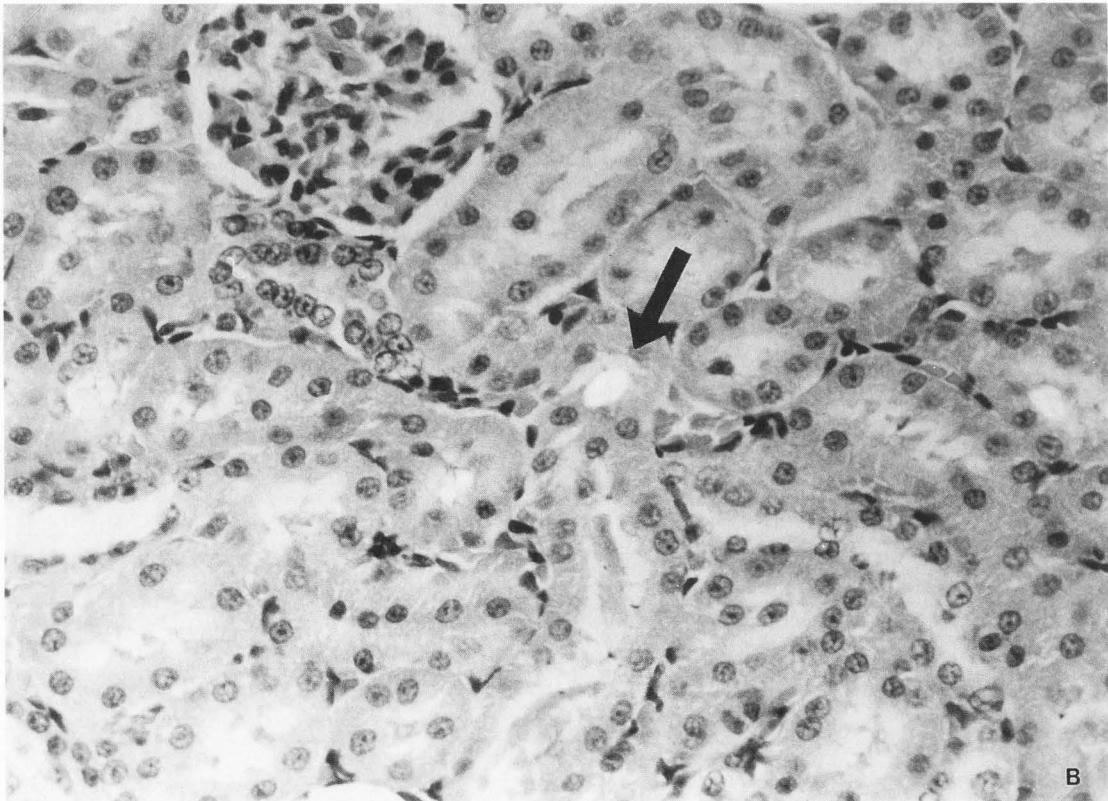
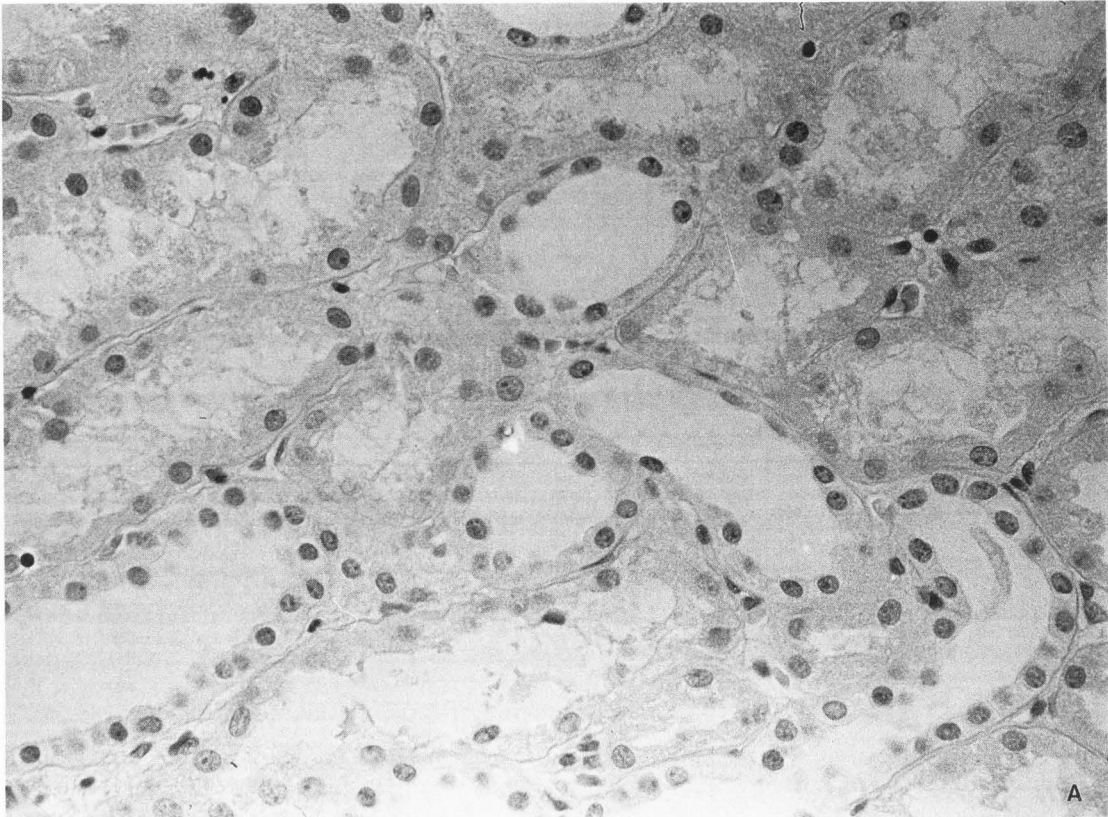
crystals, while treatment with exogenous GAG significantly reduces crystal adhesion [4, 17, 18]. We observed in the "static model system," that GAGs significantly reduced the cellular association of crystals. Although the mechanism of the phenomenon requires further study, we speculate that GAGs might 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 and Toback [10] also noted that diverse substances, including heparin, inhibited the endocytosis of COM crystals by acting on the cell surface, rather than on the crystal. Our results suggested that human urine also had the capacity to inhibit cellular uptake of crystals, especially in macromolecular fraction. Further investigation of the factors influencing crystal-cell interactions is expected to lead to the development

of new treatment modalities in nephrolithiasis.

While crystal-cell interactions have been noted in experiments using cultured renal tubular cells *in vitro*, the significance of these interactions in stone formation *in vivo* is not well understood. The preliminary study using the rat model of nephrolithiasis revealed that the COM crystals formed within the tubular lumen were frequently adherent to the epithelial cells or were sometimes endocytosed by the cell. Recently, Khan [7] also demonstrated that the adhesion and endocytosis of COM crystals could serve as a nidus for further development of kidney stone.

Although the experimental stone model causes severe crystalluria, it is possible that some pathological or environmental factors temporarily cause the similar crystalluria in the human kidneys. Therefore, these crystal-cell interactions might occur in the stone-forming kidney and be one of the initiating processes in nephrolithiasis.

Calcium oxalate crystal - cell interactions



In conclusion, COM crystals could adhere to the intact surface of MDCK cells by some biological mechanisms, followed by endocytosis, as indicated in the "dynamic model system" mimicking the urine flow in the renal tubules. These crystal-cell interactions are suggested to be one of the initiating processes in the formation of kidney stones. Further elucidation of the mechanism and the regulatory factors of this process may provide new insight into the pathogenesis of kidney stones.

Acknowledgements

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Discussion with Reviewers

S.R. Khan: Why did the authors deem it necessary to make rats hypercalciuric by giving them a high calcium diet; 10 mg per 100 g of oxalate should, by itself, be enough to induce crystal deposition in the kidneys.
Authors: Our pilot study revealed that the intraperito-

renal injections of 10 mg of sodium oxalate per 100 g of body weight were not enough to induce crystal deposition in the kidney. Therefore, we supplemented with the high calcium diet in this study. The discrepancy between your animal model and ours might be responsible for the difference of the weight and age of the rats.

C.R. Scheid: Did you ever examine crystal adherence to confluent cultures of MDCK cells (conditions that would more closely mimic those *in vivo*)? The studies of Mandel and Riese [14] on primary cultures of renal papillary cells suggested that adherence of crystals is promoted by conditions that disrupt the monolayer. Thus, it is possible that subconfluent (and hence, non-polarized) cultures may have binding sites for crystals that would not be available in polarized epithelia.

J.C. Romijn: It is not clear why subconfluent (rather than confluent) monolayers were used in both the static and the dynamic model. Confluent layers are required to obtain cell polarization, which not only creates a more physiological condition but also is known to affect crystal cell interaction [21]. Which considerations have led to the choice of subconfluent cultures?

Authors: In our previous report, we confirmed that COM crystals could adhere to intact MDCK cells in confluent cultures [1]. Moreover, the treatment with 0.1 M HCl or the incubation with gentamicin for 24 hours reduced the number of adherent crystals, which suggested that cell injuries might not be essential for the attachment of microcrystals to the tubular cells, in contrast to the observation by Mandel and Riese [14]. We used subconfluent cultures in this study to make it easy to assess the cellular uptake of crystals in a quantitative and morphological fashion. We reconfirmed that most of the cells used in this study contacted to each other, differentiated well and had no morphological differences from the cells in completely confluent cultures under phase-contrast microscopy and SEM. However, the relationship of this study using subconfluent cultures to the events in the intact renal tubules are still uncertain.

C.R. Scheid: You carried out your binding studies in the presence of 3% serum. Did you ever check the effects of serum on crystal binding?

Authors: Our pilot study revealed that the serum-free medium could not keep the cell viability during the incubation with COM crystals, so we used MEM containing 3% FCS. In our pilot study, FCS were demonstrated to promote the cellular uptake of crystals. We suppose it might be responsible for increased cell viability.

C.R. Scheid: When you examined the effects of urine on crystal binding, did you always add the crystals in the urine (i.e., under conditions in which crystals may have

been coated with macromolecules)?

Authors: In the "dynamic model system," we suspended COM crystals in the urine samples and immediately dipped MDCK cells in this suspension to evaluate the effect of human urine on cellular uptake of crystals. The result suggested that the human urine had the capacity to inhibit cellular uptake of crystals. However, it has not been well understood how the urinary macromolecules inhibit the phenomenon. Do the macromolecules act on the crystals, on the cells or on both of them? We now speculate that the inhibitors in the urine might act not only on the crystals (as had been considered in the chemical studies) but also on the cells.

Reviewer III: How do the authors envision that if cells in the intact kidney endocytose crystals, as do MDCK cells in culture, that this leads to kidney stone formation?

Authors: We have already reported that endocytosis of COM crystals stimulated cell multiplication [9]. Crystal-induced cellular proliferation might expose new domains on the plasma membrane or cause the disturbance of the tubular epithelium, which provides the calculus nidus for further crystal binding and anchored crystal aggregation and growth in the supersaturated urine. In addition, we speculate that crystal-associated cells are released from tubular epithelium as crystal-cell complexes, which might occlude the tubules and cause further crystal retention. Thus, crystal-cell interactions shown in this study are supposed to promote the aggregation and growth of crystals as the initial process of kidney stone formation.

Reviewer IV: What are typical GAG levels in the urine, and how does this relate to your observations in Figure 5? Although there may be some statistical significance, the physiologic significance is unclear.

J.C. Romijn: In our experiments, substances like SPP already showed 50% inhibition at μM concentrations. Alternatively, could the presence of FCS during incubation have an effect on crystal surface properties?

K. Suzuki: Although you stated the significant inhibition of SPP, ChS and heparin, were there any differences when they were compared at normal urinary concentration?

Authors: Concentrations of GAGs used in this study were higher than those in normal urine, and the effects were modest, although they were significant. We suppose that these might be responsible for the presence of 3% FCS during incubation, which were demonstrated to promote the cellular uptake of crystals in our pilot study. Since the renal epithelium is always exposed to some of the GAGs in the urine, it is possible that crystal-cell interactions in the renal tubules are suppressed to some ex-

tent by urinary GAGs. On the other hand, it is not known what substances (macromolecules) are responsible for the inhibitory activities of human urine. We speculate that not only GAGs but other macromolecules might have the inhibitory activities, and we are now pushing the research forward.

J.C. Romijn: In this work, different models were used to study the interactions between renal cells and COM crystals. Of these, the dynamic *in vitro* model seems to be an improvement as compared to the earlier (static) model because it is considered to create a more physiological situation by using a "flowing" crystal solution. It would be interesting to know whether this improvement actually leads to a different (i.e., more physiological) behavior in the dynamic model. Have the authors performed any comparative experiments demonstrating such a difference?

Authors: Although the static model system revealed that COM crystals could adhere to the MDCK cells and be endocytosed, it only weakly modelled conditions in the renal tubules. Based on our question as to whether these crystal-cell interactions occur even in the urine flow, we developed the dynamic model system. Scanning electron micrographs verified that the crystal-cell interaction in the dynamic model system was similar to that in the static model system. However, the flow rate appeared to have some influences on cellular uptake of crystals. We are now trying to study the effect of flow rate on crystal-cell interaction.

J.C. Romijn: In the present paper, the static model was mainly used to determine crystal internalization, whereas the dynamic model was applied to assess primarily crystal attachment. What is the rationale for this distinct use of the two models?

Authors: In the static model system, "cellular uptake of crystals" implies both crystal adhesion and endocytosis, as shown in Figures 1 and 2. The effect of human urine on crystal-cell interaction was assessed by measurement of calcium content immediately after 5 minutes reaction in the dynamic model system, which was considered to evaluate mainly crystal adhesion. Since we speculated that the crystal-cell interaction might be influenced at its first process (crystal adhesion), we began with the assessment of the effect of human urine primarily on crystal adhesion.

J.C. Romijn: While it is suggested in this paper that GAGs inhibit crystal-cell interaction by coating the cell surface, results from our laboratory and recent data published by Lieske *et al.* [24] in fact demonstrate that protection against adherence is mediated by coating of crystals rather than of the cells. Have the authors excluded the possibility that, due to incomplete washing, sufficient amounts of GAGs have remained after the preincubation step to inhibit subsequent crystal binding?

Authors: First, we speculated that GAGs might possibly reduce the cell capacity to adhere to and internalize the crystals. However, in our experiment about the effect of GAGs on cellular uptake of crystals, it could not be denied that remaining GAGs due to incomplete washing might also inhibit the cellular uptake of crystals by acting on the crystals. Both possibilities, that GAGs might act on the cells or on the crystals, still exist and require further study.

K. Suzuki: Did you check and examine the effect of pH and osmolarity of the solution (culture medium, GAGs and urine)?

Authors: All solutions used in this study were adjusted to pH 7.4. But we did not check the effect of pH and osmolarity on crystal-cell interaction. Recently, a paper by Verkoelen *et al.* [25] demonstrated that a lower pH predisposed the tubular cells for crystal attachment.

Reviewer IV: In Materials and Methods, the authors state: "The cells were transferred to a poly-L-lysine (Sigma) coated glass piece." Is a "glass piece" the same as a cover slip?

Authors: We cut the slide glass (76 mm x 26 mm, thickness 10 mm) into small pieces (8 mm x 8 mm). These "glass pieces" were used for observing trypsinized cells with SEM as described in the text.

Additional References

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