Research Article

Active Phagocytosis and Diachronic Processing of Calcium Oxalate Monohydrate Crystals in an *in vitro* Macrophage Model

Atsushi Okada^a Hiromasa Aoki^b Daichi Onozato^b Taiki Kato^a
Tadahiro Hashita^b Hiroshi Takase^c Teruaki Sugino^a Rei Unno^a
Kazumi Taguchi^a Shuzo Hamamoto^a Ryosuke Ando^a Kentaro Mizuno^d
Keiichi Tozawa^a Tamihide Matsunaga^b Kenjiro Kohri^a Takahiro Yasui^a

^aDepartment of Nephro-Urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; ^bDepartment of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan; ^cCore Laboratory, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; ^dDepartment of Pediatric Urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Keywords

Calcium oxalate · Kidney · Macrophages · Nephrolithiasis · Phagocytosis

Abstract

Background: We previously discovered that renal macrophages (Mφs) phagocytose renal calcium oxalate monohydrate (COM) crystals. This study investigated the processing of engulfed crystals using *in vitro* models. **Methods:** J774.1 mouse Mφs were exposed to COM crystals and observed for 24 h using polarized light microscopy with/without cytochalasin B (CB), an inhibitor of phagocytosis, to confirm active crystal phagocytosis. LysoTracker and immunohistochemical staining using transmission electron microscopy for lysosomal-associated membrane protein 1 were used to confirm engulfed COM crystal uptake into lysosomes. Diachronic tracking of specific Mφs was performed to capture the entire course of engulfed COM crystal processing using polarized light microscopy. Follow-up studies of fluorescent COM (f-COM) crystals using imaging cytometry were performed in the presence and absence of nigericin to dissipate the pH gradient in acidic organelles. **Results:** Phagocytosis rates increased with COM density and were significantly lower in cells treated with CB ($p < 0.01$). We observed that engulfed crystals colocalized within lysosomes of the Mφs; moreover, diachronic observation indicated that the engulfed COM crystals were subdivided during Mφ division and eliminated by the 7th day of culture. Additionally, imaging cytometry showed that the fluorescence level of f-COM crystals in the nigericin (–) group after 48 h was significantly lower than that in the nigericin (+) group. **Conclusions:** This study confirmed active phagocytosis and lysosomal processing of engulfed COM crystals by Mφs. This discovery is expected to contribute to the development of future drugs that enhance the COM crystal phagocytic ability of Mφs.

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Atsushi Okada MD, PhD
Department of Nephro-Urology
Nagoya City University Graduate School of Medical Sciences
1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467–8601 (Japan)
E-Mail a-okada@med.nagoya-cu.ac.jp

Introduction

Clinical dissolution therapies for the cystine and uric acid components of kidney stones have been used for >50 years [1, 2]; however, dissolution therapy for calcium oxalate crystals, which represent the majority of stone components, is not currently available.

The spontaneous elimination of renal intratubular calcium oxalate monohydrate (COM) crystals was previously investigated using a hyperoxaluric mouse model established by our laboratory [3]. To examine the gene-expression profiles in stone-forming mouse kidneys, we subsequently performed microarray analysis and identified characteristic changes in macrophage (Mφ)-related inflammation gene groups using clustering, gene ontology, and pathway analyses [4]. Additionally, ultrastructural and immunohistochemical observations of the mouse kidneys demonstrated migration and crystal phagocytosis in renal interstitial Mφs [5].

Following our discovery of the “stone elimination phenomenon,” several reports were published suggesting morphological disappearance of the renal crystals in vitro [6], as well as in rats and humans [7]. Therefore, this study elucidated the processing mechanisms of engulfed COM crystals using in vitro Mφ models with the goal of improving treatment options for patients suffering from kidney stones.

Materials and Methods

Culture of J774.1 Cells

J774.1 mouse cultured Mφ cells (Japanese Collection of Research Bioresources Cell Bank, Tokyo, Japan) were cultured in Dulbecco's modified Eagle medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% sterile fetal bovine serum (Hana-Nesco Bio Corp., Tokyo, Japan), 60 µg/mL ampicillin sodium (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 50 µg/mL kanamycin sulfate (Meiji Seika Pharma Co., Ltd.). Cells were split to between 0.5×10^5 cells/mL and 1×10^5 cells/mL every third or fourth day using 25 cm² cell-culture flasks (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) in a 5% CO₂ incubator at 37 °C.

Preparation of COM Crystals

Preparation of COM crystals was performed according to a previous report [9]. Briefly, oxalic acid sodium (200 mM, 0.5 mL) and calcium chloride (200 mM, 0.5 mL) were mixed in a buffer containing 90 mM Tris-HCl and 10 mM NaCl (pH 7.4) at room temperature (RT; 20 °C) to a final concentration of 10 mM. The COM crystals were equilibrated for 3 days, washed 3 times with sodium- and chloride-free distilled water, saturated with calcium oxalate, and resuspended to a final concentration of 2.92 mg/mL, followed by adjustment to pH 6.8.

Preparation of the Cell Suspension

J774.1 cells were centrifuged at RT (20 °C) at 190 *g* for 8 min, the supernatant was removed by decanting and discarded, and cells were collected as pellets. Following the addition of 10 mL culture medium, cells were centrifuged as described previously, and pellets were collected. Subsequently, 5 mL culture medium was added to the cell pellets, 200 µL of each suspension was transferred to a 1.7 mL centrifuge tube (Nippon Genetics Co. Ltd., Tokyo, Japan), and cell counts and survival rates were determined. When the survival rates exceeded 90%, the cell suspensions were used for subsequent examination. The cell numbers and concentrations required for each experiment were established based on the measured cell counts.

Phagocytosis of COM Crystals

J774.1 cells were prepared as cell suspensions at 5×10^5 cells/mL, placed in an 8 chamber polystyrene vessel tissue culture-treated glass slide (Becton, Dickinson and Co.) at 200 μ L/well, and incubated for 1 h. The phagocytosis inhibitor cytochalasin B (CB; Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium to a final concentration of 10 μ g/mL. After a 30-min incubation, prepared COM crystals were added to the culture medium at 12.5 or 62.5 μ g/cm². At 24 h following the addition of the COM crystals, the glass slides were shaken using a plate shaker for 10 s, the supernatant was removed, and the slides were washed twice with 400 μ L phosphate-buffered saline (PBS[–]; pre-warmed to 37 °C; Sigma-Aldrich), from which magnesium and calcium had been removed to avoid influence on the COM crystals. After removal of the wash solution, 200 μ L of fresh PBS(–) containing 0.25% trypsin was added to the slide, which was subsequently placed at RT (20 °C) for 30 min. Culture medium (800 μ L) with serum was added to the cell suspension to stop trypsin digestion, and collected cells were then centrifuged at 2,000 *g* for 5 min, followed by removal of the supernatant.

The cell solutions were then supplemented with 200 μ L PBS(–) containing 4% paraformaldehyde and fixed at RT for 30 min. The fixed cells were centrifuged at 2,000 *g* for 5 min, and 10 μ L aliquots of the fixed cell suspensions were transferred to a Burkert-Turk hemocytometer (Hirschmann EM Techcolor; Hirschmann GmbH, Fluorn-winzeln, Germany). The number of intracellular COM crystals was measured using a microscope (Olympus Model CHA; Olympus, Tokyo, Japan) with a 40 $\times$ objective lens. The COM crystals were counted in 2 locations on the hemocytometer, and the results were used when the total cell count exceeded 100 for each site. Based on these values, phagocytosis rates were calculated as the ratio of cells containing >1 COM crystal relative to the overall cell number. These studies were conducted at each COM concentration and in each group (*n* = 3).

Transmission Electron Microscopy

J774.1 cells were seeded in 6 well plates (1×10^5 cells/well) in DMEM containing 10% fetal bovine serum + 1% penicillin/streptomycin. After overnight incubation, cells were exposed to COM crystals at 62.5 μ g/cm² for 24 h, and samples were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C. After fixation, the specimens were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 45 min and subsequently dehydrated in a graded series of ethanol and embedded in epoxy resin.

Ultrathin sections were cut using an ULTRACUT-S (Leica, Wetzlar, Germany) with a diamond knife and stained with 2% uranyl acetate in distilled water for 15 min, followed by staining with a lead staining solution for 5 min. Sections were examined using a JEM-1400 plus (JEOL, Tokyo, Japan) electron microscope at 100 kV.

Immunohistochemical Staining for a Lysosome Marker using Transmission Electron Microscopy

Following the exposure of COM crystals at 62.5 μ g/cm² on J774.1 cells for 24 h, the cells were washed with PBS for 1 min and fixed with PBS containing 4% paraformaldehyde for 10 min at RT (20 °C). After washing 3 times with PBS, the following membrane permeation treatments were sequentially performed: freeze and thaw 3 times, tris-buffered saline (TBS) containing 25 μ M digitonin for 5 min, TBS containing 10 μ M digitonin for 5 min, freeze and thaw 2 times, TBS containing 25 μ M digitonin for 10 min, and TBS containing 10 μ M digitonin for 10 min. After washing 3 times with TBS, 100 μ L of blocking solution (TBC containing 20% of Block Ace (Dainipponseiyaku, Suita, Japan) and 0.3 M glycine) was added for 30 min at RT.

The cells were then incubated overnight with polyclonal antilyosomal-associated membrane protein 1 rabbit IgG (abcam Co., Ltd., Gunma, Japan) 300 fold diluted in TBS solution containing 20% of Block Ace at 4 °C. After washing 3 times with TBS, the secondary

antibody, anti-IgG, Rabbit, Goat-Poly, Nanogold, ϕ 1.4 nm (Nanoprobes, Inc., Yaphank, NY, USA), 100 fold diluted TBS containing 20% of Block Ace were added for 2 h at RT (20 °C). After washing 5 times with TBS for 3 min, the signals were confirmed by fluorescence microscopy.

Subsequently, the cells were prefixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 30 min. After washing 3 times with distilled water for 3 min, the samples were reacted with Silver Enhancement Kit HQ Silver (Nanoprobes, Inc.) for 6 min. After washing 3 times with distilled water for 3 min, the samples were reacted with 0.05% chloroauric acid (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 2 min. After washing 3 times with distilled water for 2 min, the samples were postfixed with 0.1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 30 min and subsequently dehydrated in a graded series of ethanol and embedded in epoxy resin. Thereafter, ultrathin sectioning and observations were performed in the same manner as for transmission electron microscopy (TEM).

Staining of COM Crystal-Engulfed Cells with LysoTracker

Preparation of the J771.1 cell suspension and COM crystal exposure were performed as described above. After incubation for the stated period, cells were washed with 2 mL PBS(–) and warmed to 37 °C, followed by the addition of 2 mL culture medium containing 50 nM LysoTracker Red DND-99 (Invitrogen, Carlsbad, CA, USA) [8]. Following a 90-min incubation, the cells were washed with medium and then with 2 mL PBS(–). After aspirating the PBS and the addition of 1 mL fresh PBS(–), the cells were observed by light field, polarization, and fluorescence microscopy using an Eclipse TE2000-E confocal microscope (Nikon, Tokyo, Japan) with green excitation as the fluorescence filter.

Diachronic Observation of Engulfed COM Crystals

J774.1 cells were prepared in suspension at 5×10^5 cells/mL and placed into 12 well plates at 1 mL/well. After a 1-h incubation, COM crystals were added to the culture medium at 12.5 μ g/well. After 24 h, the 12 well plates were shaken for 10 s, the medium was removed using an aspirator, and the plates were washed twice with 1 mL PBS(–) that had been warmed to 37 °C. After removal of the PBS(–), 700 μ L of fresh PBS(–) containing 0.25% trypsin was added, and the reaction was allowed to occur at RT (20 °C) for 30 min. The cells were then separated from the plate surface by pipetting, and the resulting cell suspension was transferred to 1.7-mL tubes containing 700 μ L culture medium with serum to stop the trypsin digestion. After centrifugation at 2,000 g for 5 min, 1 mL PBS(–) was added, and samples were centrifuged for 5 min at 2,000 g , and the supernatant was removed. Subsequently, 500 μ L of culture medium containing serum was added, and cells were counted using 10 μ L of the sample. Cell suspensions at 2×10^3 cells/mL were prepared and added to 35 mm glass-base dishes (Iwaki; Asahi Glass Co., Ltd., Tokyo, Japan) at 2 mL/well. Using a confocal microscope, diachronic observation of the cells was performed using a light field and polarization.

Follow-Up Analysis of Fluorescent COM Crystals Phagocytosed by M ϕ s Following Inhibition of Intracellular Acidification

To capture the disappearance of COM crystals phagocytosed by J774.1 cells, we used the intracellular acidification inhibitor nigericin. COM crystals were fluorescently labeled according to the method of Chaifar et al. [10]. Briefly, 5 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ was mixed with 0.5 mM $\text{Na}_2\text{C}_2\text{O}_4$ in a buffer containing 90 mM Tris-HCl and 10 mM NaCl (pH 7.4). The solutions were incubated at 25 °C overnight, and COM crystals were harvested by centrifugation at 2,000 g for 5 min. The supernatant was discarded, and COM crystals were resuspended in methanol. After another round of centrifugation at 2,000 g for 5 min, the methanol was discarded, and the crystals were air-dried overnight at RT (20 °C). For staining/labeling, COM

crystals were crystallized in the presence of 0.11 mg/mL AlexaFluor 488 (Thermo Fisher Scientific, Burlington, ON, Canada). The prepared fluorescent COM (f-COM) crystals were capable of identification at an excitation wavelength of 488 nm and an emission wavelength of 519 nm was prepared.

J774.1 cells were cultured to semi-confluence with DMEM + penicillin/streptomycin medium in a 10 cm Petri dish, followed by the addition of 62.5 µg/mL f-COM crystals and incubation for 24 h. After washing twice with PBS, 4×10^4 cells/well were harvested in 60 wells of a 96 well plate coated with 0.1% gelatin and incubated at 4 °C overnight. In 30 of the wells, 2 µmol/L nigericin [11], a protonophore that can be used to dissipate the pH gradient in acidic organelles, was administered (the nigericin[+] group), with the remaining 30 wells representing the nigericin(–) group. Thereafter, live cell imaging was performed using In Cell Analyzer 6000 (GE Healthcare UK Ltd., Buckinghamshire, England), to quantify the fluorescence emission of AlexaFluor 488 during incubation under the condition of 5% CO₂ and 37 °C. The fluorescence of f-COM crystals per cells in the 60 wells was scanned simultaneously, and sequential image capture of the wells was performed every 20 min for 48 h (2,880 min). After the scanning of all images, each fluorescence intensity was shown as quantified values, the average value at the 0 min time point in each group was set to 1, and the value at each time point was expressed by a ratio.

Detection of Internalization of f-COM Crystals in Mφ

This observation was performed to confirm that the fluorescence data obtained in the follow-up analysis of f-COM crystals using imaging cytometry reflected that of COM crystals intrinsic to Mφ. Culture of J774.1 cells to semi-confluence and addition of f-COM crystals were performed in the same manner as for the follow-up analysis. The cells were fixed with PBS containing 4% paraformaldehyde at RT (20 °C) for 10 min. After washing with PBS for 5 min, 0.1% Triton X-100 in PBS was administered at RT (20 °C) for 3 min. The cells were stained with Acti-stain TM 555 phalloidin (Cosmo Bio USA, Inc., Carlsbad, CA, USA) and 4,6-diamidino-2-phenylindole, dihydrochloride (Cosmo Bio USA) at RT (20 °C) for 30 min. After washing with PBS, the samples were scanned using the In Cell Analyzer 6000.

Statistical Methods

For the phagocytosis assay, differences were assessed using a Student *t* test. Results were considered statistically significant at *p* < 0.05. Analyses were performed using JMP version 12.2.0 (SAS Institute, Inc., Cary, NC, USA).

Results

Phagocytosis Assay of COM Crystals with or without CB

We observed that phagocytosis rates increased according to the exposed COM density (19.3% at COM 12.5 µg/cm² and 39.7% at COM 62.5 µg/cm²) and were significantly suppressed by CB administration (0.7% at COM 12.5 µg/cm² and 13.3% at COM 62.5 µg/cm²; Fig. 1).

Evidence for Localization of Phagocytosed COM Crystals in Lysosomes of Mφs

Using phase-contrast microscopy and polarized light microscopy, the majority of COM crystals after 24 h of exposure were observed at the same position as Mφs. Moreover, using fluorescent images, almost all LysoTracker fluorescence colocalized with the polarization of the COM crystals (Fig. 2). Additionally, TEM analysis identified COM crystals in structures surrounded by a single membrane exhibiting expression of lysosomal-associated membrane protein-1, which identified the organelles as lysosomes (Fig. 3).

Fig. 1. Phagocytosis rates of COM crystals in J774.1 cells. Phagocytosis rates were calculated as the number of phagocytic cells/total cell number (%). The J774.1 cells had a phagocytosis rate of $19.3 \pm 5.9\%$ for COM at $12 \mu\text{g}/\text{cm}^2$. Administration of the phagocytosis inhibitor CB significantly decreased the rate to $0.7 \pm 1.2\%$ ($p = 0.0056$). Additionally, the rate for COM at $62.5 \mu\text{g}/\text{cm}^2$ ($39.7 \pm 5.51\%$) was significantly decreased to $13.3 \pm 3.21\%$ through the administration of CB ($p = 0.002$). CB, cytochalasin B; COM, calcium oxalate monohydrate.

Fig. 2. LysoTracker assay for COM crystal-containing Mps. **A** Phase-contrast microscopy, **(B)** polarized light microscopy, and **(C)** fluorescent image (magnification, 400 $\times$). **D** Merged image of **(B, C)**. On day 1 after exposure of J774.1 cells to COM crystals, almost all LysoTracker fluorescence colocalized with the polarization of the COM crystals.

Fig. 3. TEM and immune-TEM of COM crystals phagocytosed by J774.1 cells. COM crystals were identified in structures surrounded by a single membrane. **A** Magnification, 1,500× (scale bar: 5.0 μm). **B** Magnification, 5,000× (scale bar: 1.0 μm). The arrows show COM crystals in single membrane organelles. **C, D** Showed immune-TEM images. **C** Magnification, 1,500× (scale bar: 5.0 μm). **D** Magnification, 5,000× (scale bar: 1.0 μm). Silver-enhanced gold particles indicating anti-LAMP-1 antibody are localized on the single membrane of lysosomes.

Diachronic Observation of Engulfed COM Crystals

Using polarized light microscopy, diachronic observation of the specific crystals revealed that many phagocytosed crystals were fragmented by the division of Mφs and were not visible on day 6 (Fig. 4a–c), although some intracellular crystals were not divided or diminished (Fig. 4c).

Follow-Up Analysis of f-COM Crystals Phagocytosed by Mφs Following Inhibition of Intracellular Acidification

J774.1 cell incorporation of scattered peripheral crystals resulted in increased levels of f-COM fluorescence, resulting in equivalent levels in cells of both nigericin (+) and nigericin (–) groups up to 8 h after treatment. Thereafter, the level of f-COM fluorescence gradually

Fig. 4. Diachronic observation of engulfed COM crystals. J774.1 cells disseminated in a 35-mm glass-base dish were observed using the polarized mode of the confocal microscope. Specific cells containing COM crystals were observed at 1, 3, 4, 5, and 6 days after dissemination. White arrows show intracellular COM crystals that had distributed with cell division (**A–C**). Red arrows show intracellular COM crystals that did not separate or were diminished (**C**). (Scale bar: 100 μ m).

decreased; however, after 48 h, the nigericin (+) group returned to levels observed at the beginning of the experiment. Conversely, fluorescence in the nigericin(–) group significantly decreased to approximately 35% of the initial fluorescence levels after 48 h (Fig. 5; online suppl. Data 1; for all online suppl. material, see www.karger.com/doi/10.1159/000501965).

To confirm the internalization of f-COM in M ϕ s, multiple fluorescently stained M ϕ s were observed using an In Cell Analyzer 6000. The results revealed that f-COM crystals were present at the same site as the cytoplasm (stained red by phalloidin) and the M ϕ s (stained blue by 4,6-diamidino-2-phenylindole, dihydrochloride; Fig. 6).

Discussion

Our previous studies using a hyperoxaluric nephrolithiasis model revealed the possibility of crystal processing by M ϕ s [3–5]. Morphologically, interstitial M ϕ s of the kidney were found to surround calcium oxalate crystals in hyperoxaluric rat models [12]. These crystal-engulfing M ϕ s produced the cytokine interleukin-6 [13]. Additionally, Kusmartsev et al. [14] reported calcium oxalate stone fragment and crystal phagocytosis by human M ϕ s. Furthermore, we recently elucidated the gene-expression profiles of human Randall's plaque tissues, with subsequent analysis of gene networks demonstrating that these plaque tissues were associated with upregulated genes, including a relationship between M ϕ activation and

Fig. 5. Follow-up analysis of f-COM crystals phagocytosed by Mφs following inhibition by intracellular acidification. The vertical scale represents the ratio of the fluorescence of AlexaFluor 488 bound to the calcium oxalate crystals existing in the cell in the 2-dimensional observation. The average value at the 0 min time point in each group was set to 1, and the value at each time was expressed by a ratio. A significant difference ($p < 0.05$) according to a t test was observed between groups from 300 to 2,880 min.

Fig. 6. Internalization of f-COM in Mφs using multiple fluorescently stained Mφs. The fluorescence-stained COM crystals of AlexaFluor 488 are shown as green, phalloidin-stained Mφs cytoplasm as red, and DAPI-stained Mφ nuclei as blue. The f-COM crystals are present at the same site as the cytoplasm (stained red by phalloidin) and the Mφs (stained blue by DAPI). Right: magnified image of boxed area.

proinflammatory cytokines [15]. Based on these results, it was suggested that calcium oxalate crystal phagocytosis was likely to occur within kidney tissue.

Conversely, Mφs have been classically recognized as having a role in kidney stone formation. In *in vitro* studies by Umekawa et al. [16, 17], the exposure of renal tubule cells to calcium oxalate crystals promoted the production of monocyte chemoattractant protein-1, suggesting that inflammation is mediated by Mφs. The similarities between the mechanisms involved in the formation of atherosclerosis and kidney stones also suggest a common inflammatory etiology. For example, susceptible ages include males of middle and old age and postmenopausal female; another cause is a westernized diet. Components include calcium and osteopontin, and both are induced by cytokines and Mφs. The grounds for such theories are based on results from our previous studies, including the correlation between aortic calcification index and the prevalence of kidney stones [18], the positive association between kidney stone formation and conventional risk factors of coronary heart disease (as deter-

mined by a large-scale cohort study) [19], an in vitro study using cocultured Mφs with renal epithelial cells and adipocytes [9], and an in vivo study using metabolic syndrome model animals [20]. We were also inspired by a study on the regression of atherosclerotic plaques [21], and ultimately considered that Mφs might serve as a therapeutic target, potentially leading to the dissolution of kidney stones. Notably, the early phase of atherosclerotic plaque regression showed a loss of foam cells from the lesions and a concomitant increase in nonfoam cell (healthy) Mφs surrounding areas of necrosis, in which removal of the material by an influx of functioning, healthy phagocytes was observed to occur [22]. In addition, we recently reported that kidney stone formers had more renal parenchymal crystals than nonstone formers, particularly in the papilla region, and the relationship between the crystal and Mφ number [23]. The differences in the crystal processing ability of Mφs between healthy and stone-forming patients should be further studied.

In subsequent studies, we focused our attention on the polarity of Mφs as a potential explanation for the possibility that they might function during both the promotion and suppression of kidney stone formation. Using anti-inflammatory-type Mφ (M2)-deficient mice, we demonstrated that M2 cells could suppress calcium oxalate nephrolithiasis [24]. Furthermore, using an obese mouse model of metabolic syndrome, inflammatory-type (M1) Mφs were shown to facilitate renal crystal deposition [25]. Additionally, we investigated the role of M1/M2 cells in crystal development by transfusion and M1/M2 Mφ induction, concluding that renal crystal development was facilitated by M1 Mφs but suppressed by M2 Mφs [26]. Based on these results, we proposed the hypothesis that humans are able to remove renal crystals via M2 Mφs, which naturally protect the kidney from clinical stone formation. In contrast, M1 Mφs activated by inflammatory environments, such as that occurring during metabolic syndrome, promote kidney stone formation. Moreover, we recently performed multiplex analysis of human urine and showed that patients with calculi exhibited lower levels of IL-4, an inducer of M2-Mφ, than those of healthy individuals [27].

We also reported the phenomenon of crystal phagocytosis by Mφs [24, 26], although the detailed mechanism of crystal processing is not yet understood. In the present study, we investigated several issues that could contribute to the development of pharmaceutical strategies for crystal processing. Such issues included whether the process of crystal phagocytosis is active or passive, whether the utilized cultured cells exhibit phagocytosis, what conditions would expose engulfed crystals to Mφs, and whether the engulfed crystals could eventually be dissolved.

To examine these issues, we utilized cytochalasins, which bind to actin filaments and inhibit polymerization and elongation. In particular, cytochalasins B inhibit phagocytosis in neutrophils and Mφs. Therefore, pharmacological inhibition by cytochalasin leads to material transport within Mφs instead of phagosome formation. Accordingly, the results of the phagocytosis assay with cytochalasins (Fig. 1) indicated that COM crystal transport within Mφs is dependent upon active phagocytosis.

Generally, the materials transported within Mφs are confined to phagosomes; subsequently, phagosomes fuse with lysosomes that contain hydrolases for digestion of the internal materials [28]. We monitored engulfed COM crystals by polarized light microscopy, fluorescence microscopy, and TEM and detected their cellular position using LysoTracker (Fig. 3). Morphologically, the crystals were shown to exist within lysosomes of Mφs (Fig. 4) and expected to be exposed to the acidified lysosomal environment. The results further indicated that COM crystals could be transported within Mφs and were subsequently surrounded by phagosomes that fused with lysosomes. Calcium oxalate stones are likely generated under acidic urinary conditions (pH 5.0–6.0) and cannot be dissolved physiologically by urine. However, some studies demonstrated that calcium oxalate could be dissolved in environments of pH 2–4 [29] and that intra-phagolysosomal conditions are likely suitable for dissolution.

The possibility of crystal phagocytosis has been reported by several groups [7, 12, 14], as well as by our group [3–5, 24, 26]; however, actual elimination of engulfed COM crystals had not been previously detected. In the present study, we presented the first evidence of diachronic elimination of engulfed COM crystals in viable Mφs using glass-base dishes and reductions in the fluorescence intensity of f-COM crystals in Mφs by imaging cytometry. Specifically, the f-COM study showed significant inhibition based on decreased fluorescence levels following administration of nigericin. This phenomenon is consistent with renal crystal elimination associated with interstitial Mφ migration that occurs in the hyperoxaluric mouse model [5], as previously reported by our group.

There are several limitations to this study. Cultured J774.1 Mφ cells were considered suitable for phagocytosis studies, as reported previously [8], although related gene expression has not been evaluated. Based on the results of this study, we considered that such expression analyses, including examining the expression of factors, such as stone matrix protein, cytokines/chemokines, and oxidative stress markers, would be essential to confirm and expand our findings. Cytochalasin is used as a phagocytosis inhibitor, but owing to its effect on actin polymerization, other potential reasons exist for lack of uptake. This includes the ability to reduce cell motility and chemotaxis [30]. In order to verify whether cell motility in the culture vessel on the glass slide or cell interaction with the crystal was significantly inhibited, the single-cell trend needed to be captured by live imaging; however, we were unable to accomplish this. Although it is unknown whether this process is active or passive, the results clearly showed that COM crystals are phagocytosed by human and animal Mφs [14].

Overall, this study provided the first confirmation of active phagocytosis and lysosomal processing of engulfed COM crystals by Mφs. These findings might be useful for the development of therapies for the dissolution of calcium oxalate stones.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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