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Calcium phosphate stones produced by Madin-Darby canine kidney (MDCK) cells inoculated in nude mice

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Abstract The canine renal distal tubular cell line Madin-Darby canine kidney (MDCK) forms calcium phosphate microliths during a long-term culture in vitro. We identified osteopontin (OPN) and calprotectin (CPT) from a urinary stone matrix. We recently also detected the expression of OPN and CPT in MDCK cells. The relationship between the mechanism of the stone formation and these stone matrix proteins is not yet known. Here, MDCK cells were cultured and inoculated in the subcutis of nude mice. After 4, 8 and 12 weeks, the inoculated tissues were resected, fixed and immunostained with polyclonal anti-human OPN and polyclonal anti-human CPT antibodies. Some serial specimens were stained with von Kossa's procedure. MDCK cells formed some follicular formations in the subcutis of nude mice at least at 12 weeks after transplantation. At 8 weeks after the inoculation, we detected small calcium phosphate stones with MDCK cells trapped in the follicles. The cells forming the stones also expressed both OPN and CPT. The CPT expression sites coincided with the stone formation sites. We confirmed that MDCK cells inoculated in nude mice had stone-forming potential, and we speculate that OPN and CPT play important roles in stone formation by MDCK cells.

Key words Madin-Darby canine kidney (MDCK) · Calcium phosphate stone · Urinary stone matrix

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Introduction

Urinary stones consist of over 90% inorganic materials and a small amount of organic matrix. In in vitro experiments, it is possible to produce a large mass of crystals by saturation with inorganic materials alone, but no formation of stones is achieved. In view of this fact, it is generally thought that the stone matrix plays an important role in the process of stone formation. We identified osteopontin (OPN) and calprotectin (CPT) from a urinary stone matrix and detected the expression of these proteins in renal distal tubular cells of stone-forming rats [7, 8, 17]. OPN is a 44-kDa phosphorylated glycoprotein which was originally isolated from mineralized rat bone matrix [4]. OPN contains the Arg-Gly-Asp (RGD) cell-binding sequence and has a high affinity to hydroxyapatite [13]. Calprotectin (CPT) is a 36-kDa calcium-binding protein and is detected in neutrophilic granulocytes and macrophages [2].

The Madin-Darby canine kidney (MDCK) cell line was derived from the normal, adult male Cocker Spaniel kidney and expresses many characteristics of renal distal tubular cells in culture [1]. MDCK cells adhere to collagen types 1 and 4 in a magnesium ion ($Mg2+$)-dependent manner, as does $\alpha 2\beta 1$ integrin, which plays an important role in the hepatocyte growth factor/scatter factor-induced tubulogenesis and branching morphogenesis of MDCK cells in collagen gels [3]. The biosynthesis and secretion of an OPN-related 20-kDa polypeptide in the MDCK cell line was reported by Ullrich et al. [15]. They described that 70% of both the 20-kDa polypeptide and 60-kDa precursor protein was secreted in the apical medium and 23% was secreted in the basolateral medium. MDCK cells are reported to produce small calcium phosphate crystals at their basolateral surfaces in vitro [6]. The relationship between the mechanism of the stone formation and these stone matrix proteins is not yet known. In the present study we inoculated MDCK cells in the subcutis of nude mice and observed that the cells produced small calcium

phosphate stones. By using this stone formation model, we examined the roles of OPN and CPT in stone formation.

Materials and methods

Cell culture

MDCK cells (the Laboratory Products Division of Dainippon Pharmaceutical, Osaka, Japan) were seeded in 75 cm^2 tissue culture flasks (Becton Dickinson, Lincoln Park, N.J.) to which 15 ml of Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin were added, and cultured at 37°C in a 5% CO_2 and 95% air atmosphere to confluence.

Transplantation

Female nude mice (KSN nude mice, 7 weeks old) were purchased from JAPAN SLC (Hamamatsu, Japan). Confluent monolayer MDCK cells were washed once with phosphate-buffered saline (PBS) and detached with a cellscraper. Cells suspended in saline (10^7/0.1 ml) were subcutaneously injected (0.2 ml per mouse) in the backs of six nude mice. Two animals were killed under ether anesthesia 4, 8 and 12 weeks after the injection. Inoculated tissues were resected and prepared for two microscopy studies. Light microscopy specimens were immediately fixed with 10% formaldehyde solution for 24 h, dehydrated and embedded in paraffin wax. Transmission electron microscopy (TEM) specimens were fixed in glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 7.4) solution for 2 h at 4°C.

Calcium staining

Von Kossa's staining

The Von Kossa procedure was used to detect calcium phosphate in the tissues [9]. Sections 4 mm thick were deparaffinized and treated with 5% silver nitrate solution and illuminated for 60 min, then rinsed with distilled water twice for 5 min each time. They were then treated with 5% sodium thiosulfate solution for 3 min. The sections were then counterstained with Kernechtrot solution for 5 min.

Pizzolato staining

The Pizzolato procedure was used to detect calcium oxalate in the tissues [11]. Sections 4 mm thick were deparaffinized and treated with 2.5% silver nitrate in 15% hydrogen peroxide solution and illuminated for 30 min, then rinsed with distilled water twice for 5 min each time. They were then counterstained with Kernechtrot solution for 5 min.

Immunohistochemical staining

Serial sections 4 mm thick were deparaffinized and hydrated through xylenes and a graded alcohol series. They were blocked with 3% H_2O_2 in methanol for 30 min, followed by washing in 0.01 M PBS. To block non-specific binding, all sections were treated with 1% normal blocking serum (rabbit or mouse) for 30 min at room temperature. The slides were then incubated with a primary antibody: rabbit polyclonal anti-human osteopontin (OPN) [5], rabbit polyclonal anti-human calprotectin (CPT) [2] or rat monoclonal anti-mouse macrophage (BM8, BMA Biomedicals, Switzerland) for 30 min at room temperature. Antibody binding was demonstrated with a Vectastain ABC kit, Rabbit or Mouse (Vector Laboratories, Burlingame, Calif.) for paraffin sections. Signals were detected with a New Fuchsin Substrate System (DAKO, Carpinteria, Calif.) and a DAB chromogen, respectively.

Transmission Electron Microscopy (TEM)

Specimens were rinsed with fresh cold buffer, postfixed in 1% osmic acid solution for 2 h at 0°C, dehydrated in a graded series of alcohol (10 min each) and embedded in Epon 812. Peripheral parts of the specimens were trimmed under a light microscope. Thin sections were stained with 2% uranyl acetate and lead mixture and examined on a transmission electron microscope (H-7000, Hitachi, Tokyo) with 75 kV acceleration voltage.

Results

Graft survival

Inoculated MDCK cells formed some pale subcutaneous patches 1 cm in diameter immediately after the injections. Within 1 week, these patches had condensed to small nodules 1–2 mm in diameter, and these were observed for at least 12 weeks after the transplantation. The light microscope analysis showed that the MDCK cells were viable and formed some large internal fluid-filled follicles lined with epithelial monolayer sheets in the peripheral parts of the nodule. The cells were necrotic and strongly stained by hematoxylin in the central parts of the nodule. Some floating MDCK cells were also observed in the large fluid-filled follicles (Fig. 1).

Calcium stain

After 4 weeks, inoculated MDCK cells formed some large follicles around the central necrosis. The necrotic cells were stained by von Kossa's method; they showed calcium phosphate (Fig. 2a). This phenomenon can be explained as coagulation necrosis with dystrophic calcification. Eight weeks after transplantation, some MDCK cells trapped in the large follicles formed some small follicles, which contained small deposits inside (Fig. 2b). The deposits were also stained by von Kossa's method; however, the cells were not. Neither the necrotic parts nor the small deposits were stained by the Pizzolato method (data not shown).

Immunostaining

OPN was expressed strongly in inoculated MDCK cells and weakly in the surrounding stroma including the central necrosis (Fig. 3a). CPT was expressed in some small follicles, which were formed with some MDCK cells trapped in large follicles and in macrophages among the surrounding stroma. It was also weakly

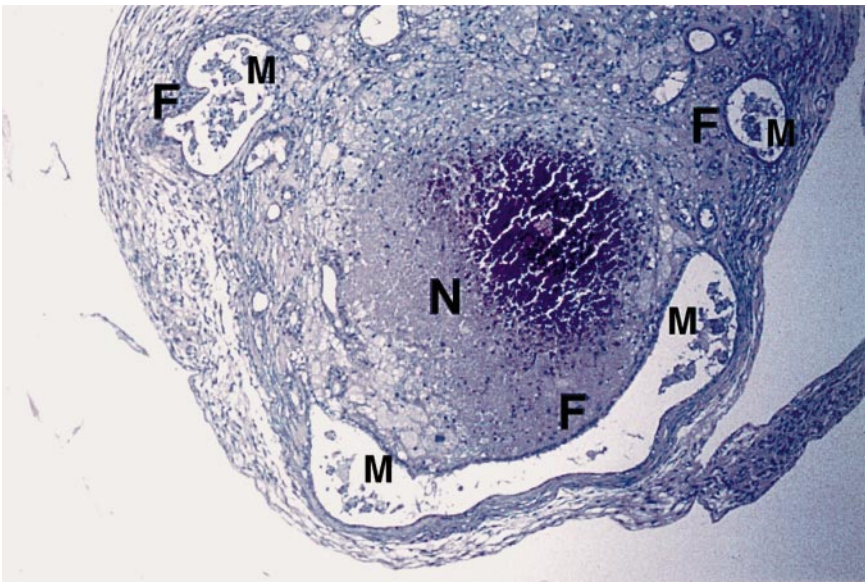

Fig. 1 Light micrograph of an MDCK nodule inoculated in a nude mouse (4 weeks after transplantation). Some large follicles (F) are shown at the peripheral position of the MDCK nodule. These follicles are lined with a monolayer sheet of MDCK cells. Some floating MDCK cells (M) are shown in the follicles. Coagulation necrosis (N) with dystrophic calcification is observed at the central position of the nodule. Magnification ×100

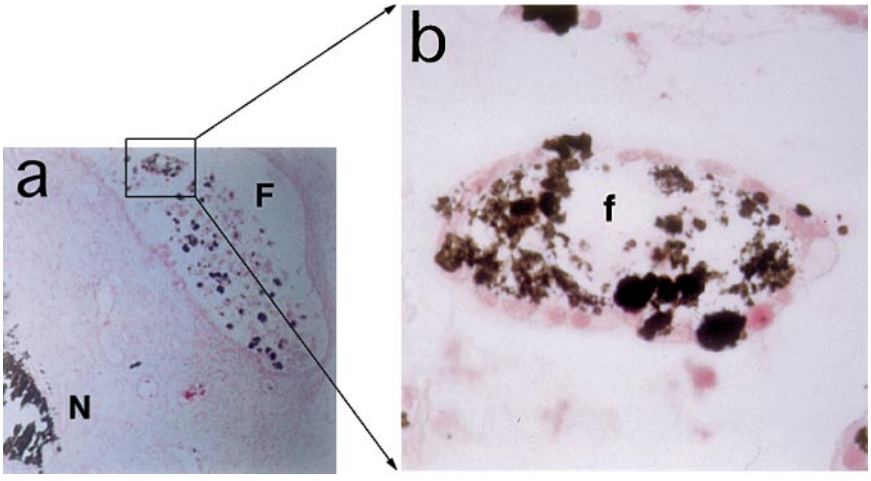

Fig. 2 Von Kossa's stain of MDCK cells (8 weeks after transplantation). Small calcium phosphate stones are observed as black deposits. These deposits are shown in small follicles floating in a large follicle. *N* coagulation necrosis, *F* large follicles, *f* small follicles.
a Magnification ×100
b Magnification ×400

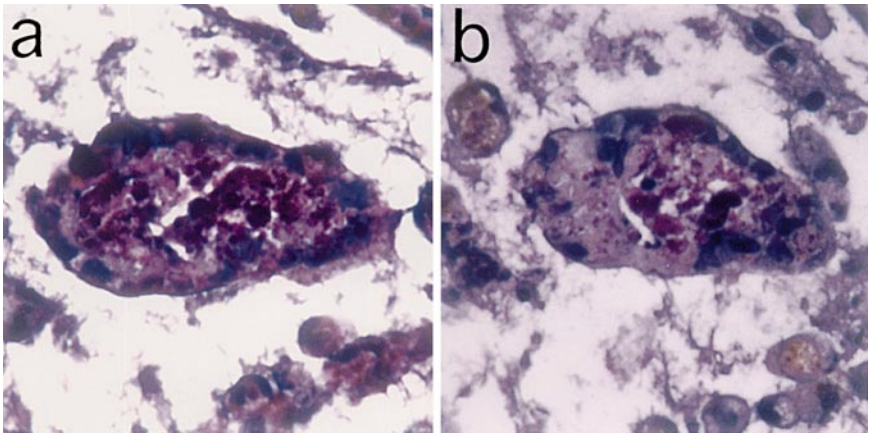

Fig. 3 Immunostaining of OPN (**a**) and CPT (**b**) in MDCK cells. MDCK cells that formed calcium phosphate stones expressed both OPN and CPT. Streptavidin ABC technique with DAB development and hematoxylin counterstain. Fig. 2b, Fig. 3a, and Fig. 3b are serial specimens. Magnification ×400

expressed in MDCK cells. The CPT expression sites coincided with the stone formation sites (Fig. 3b). Macrophages were detected only in the surrounding stroma, not in small follicles (data not shown). Table 1 summarizes the results of the immunostaining.

Transmission electron microscopy

The TEM showed that the fluid-filled large follicles were surrounded by a single layer of MDCK cells. The apical side of the cells was characterized by microvilli, which

faced the lumen of the large follicle (Fig. 4). However, some MDCK cells floating in the large follicles formed small follicles with microvilli outside (Fig. 5).

Discussion

Calcium urinary stone formation takes place in four steps: nucleation, growth, aggregation and concretion. The first three processes are observable in vitro; the concretion process is not. This means that no stone formation takes place with inorganic materials alone, suggesting that the urinary stone matrix plays an important role in the mechanism of calcium stone formation. In recent years, with the use of molecular biological techniques, the calcium urinary stone matrix has been found to consist of not only the major substances OPN and CPT, but also albumin, α-antitripsin, lysozyme and hemoglobin [16].

OPN was originally identified in the mineralized matrix of bovine bone [4], and then in other tissues such as kidneys, placenta, uterus, inner ear and also blood and milk [10]. OPN has RGD sequences, which are postulated to bind to a member of the integrin receptor family on the cell membrane. OPN also has a region consisting of nine consecutive asparitic acid residues, which is a potential region for mineral binding. CPT, the other major component of Ca-containing stone matrix, is secreted by macrophages [2] and has a potent Ca-binding capacity. Ullrich et al. [15] reported the biogenesis and secretion of an OPN-related 20-kDa polypeptide in the MDCK cell line. In the present study, we detected not only OPN but also CPT in MDCK cells.

The transplantation of MDCK cells to nude mice was originally reported by Stiles et al. [14] in 1976, and then by Rindler et al. [12] in 1979. They injected MDCK cells into baby nude mice and observed that the cells formed epithelial sheets lining internal fluid-filled glands; kidney tubule-like structures. In this study, we found that the trapped MDCK cells formed small follicles with their basolateral inside and formed calcium phosphate stones in vivo. Our findings are supported by the observation of Kageyama et al. [6] that long-term cultured MDCK cells produced small calcium phosphate crystals at their

Table 1 Results of immunostaining

	OPN	Calcium deposit	CPT	Macrophage
Inside of small follicles	+	+	+	–
MDCK cells	++	–	±	–
Surrounding stroma	±	–	+	+
Central necrosis	–	+	–	–

OPN osteopontin, *CPT* calprotectin
++ Very strong stain, + strong stain, ± weak stain, – no stain

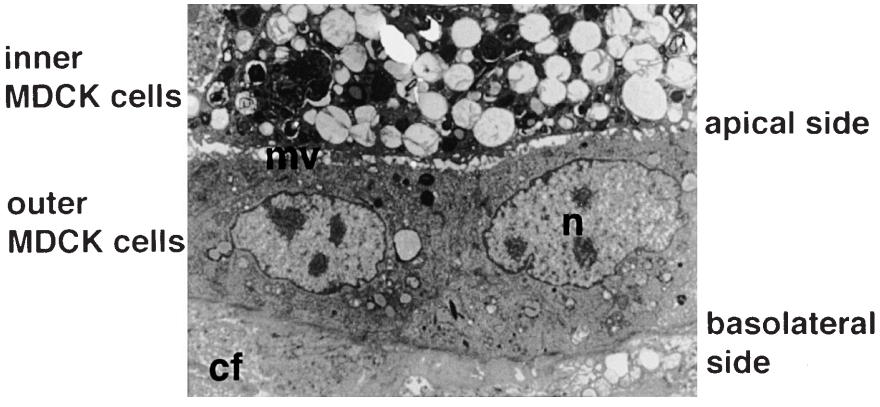

Fig. 4 TEM image of outer MDCK cells. Electron micrographs of outer MDCK cells which formed a large follicle in the subcutis of a nude mouse. Outer and inner MDCK cells contact each other on their apical surface; *mv*, microvilli, *n* nucleus, *cf* collagen fibers. Magnification ×5300

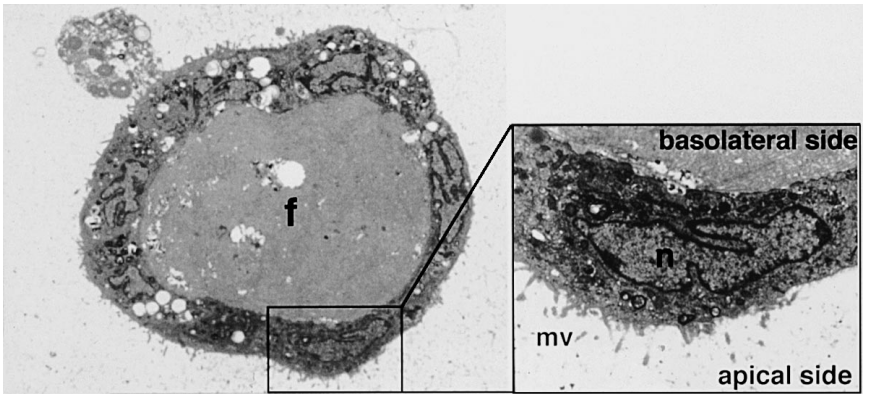

Fig. 5 TEM images of inner MDCK cells. Six inner MDCK cells trapped in a large follicle form a small follicle with their apical surface outside; *f* small follicle, *mv* microvilli, *n* nucleus. Magnification ×2000 (left) and ×5300 (right)

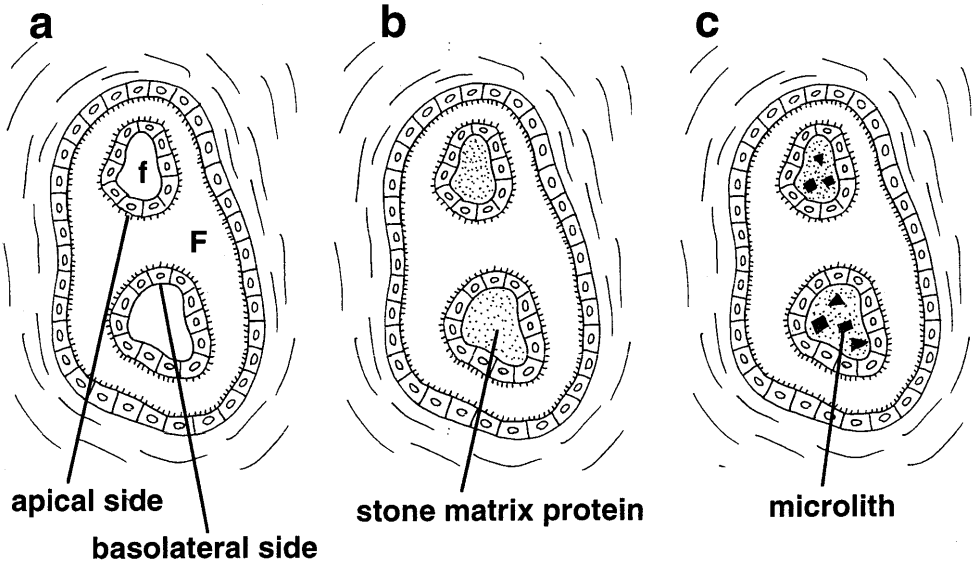

Fig. 6 Time course of microlith formation by MDCK cells. **a** Just after transplantation: MDCK cells formed outer and inner small follicles. **b** After 4 weeks stone matrix proteins are accumulated in the inner small follicles floating in the outer large follicles. **c** After 8 weeks small calcium phosphate stones are produced in the inner small follicles. *F* large follicles, *f* small follicles

basolateral surfaces in vitro. In our experimental design, we observed two types of calcification mechanism. One was coagulation necrosis with dystrophic calcification, observed at the central position of the inoculated MDCK cells, but the expression of OPN or CPT was not observed. The other was a stone-production mechanism, observed with the expression of both CPT and OPN in some small follicles formed by MDCK cells trapped in large follicles at 8 weeks. Some possible explanations of our results are as follows. MDCK cells have solute transporters on their membrane; therefore, phosphate and calcium are translated from the apical-to-basal side of the cell layer. Alternatively, or in addition, fluid is transported from the lumen of the small follicles across the epithelial layer into the lumen of the large follicles. This would also lead to increased solute concentrations in the small follicles. We suggest that trapped MDCK cells form small follicles with their basolateral surface inside and secrete OPN and CPT into the small follicles. These accumulated proteins have a calcium-binding potential, producing small calcium phosphate stones. However, large follicles are formed with their basolateral surface outside, and the secreted proteins are not accumulated or do not produce stones (Fig. 6). Macrophages were detected only in the surrounding stroma, a finding which might be related to the OPN and CPT expression in the surrounding stroma. We concluded that MDCK cells inoculated in nude mice have a stone-producing potential, and that OPN and CPT may have important roles in stone production by MDCK cells.

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