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Alendronate inhibits urinary calcium microlith formation in a three-dimensional culture model

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Abstract Osteoporosis is associated with the pathogenesis of urinary stone formation. Urinary stones are similar to bone diseases such as osteoporosis and bone metabolism in terms of pathogenesis. Bisphosphonates are potent inhibitors of bone resorption, and are used in the management of bone disease. Furthermore, bisphosphonates have a strong affinity for calcium, and a reported inhibitory effect on calcium oxalate crystallization in vitro. Thus, bisphosphonates might also inhibit urinary stone formation. Madin-Darby canine kidney (MDCK) cells form calcium phosphate microliths at the basolateral side in vitro. We investigated the inhibitory effects of new generation bisphosphonates (alendronate and incadronate) on calcium phosphate microlith formation and on the expression of osteopontin, which is an important urinary stone matrix. MDCK cells formed two types of colonies in three-dimensional soft agar culture; dark colonies containing calcium phosphate microliths and clear colonies free from microliths. We applied purified alendronate and incadronate at concentrations of 10^{-11}, 10^{-9}, 10^{-7} and 10^{-5} M to MDCK cells cultured in three-dimensional soft agar and investigated the efficiency of colony formation and the dark colony ratio (number of dark colonies relative to the total number of colonies). The administration of 10^{-9} and 10^{-7} M alendronate decreased the dark colony ratio compared with controls, whereas incadronate did not significantly alter this colony ratio compared with controls. The expression of osteopontin in cultured cells was inhibited by the 10^{-7} M alendronate administration. The present findings show that alendronate inhibits calcium stone formation, suggesting that it is effective in the prevention of urolithiasis.

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Introduction

Calcium calculi of the kidney are a frequent clinical problem. The recurrence rate without treatment for calcium oxalate renal stones is 10%, 35% and 50% at 1, 5 and 10 years, respectively [25]. Treatment for idiopathic urinary calculi has not yet proven effective, although thiazide and alkaline citrate have been effective against hypercalciuria and hypocitraturia, respectively [20].

Urinary stones contain 1–5% protein, and many reports have suggested the importance of proteins in urinary stone formation [3]. We previously extracted the proteinous fraction from calcium oxalate and calcium phosphate stones with ethylenediamine tetra-acetic acid, and identified one of the major components of urinary stones as osteopontin (OPN) [12]. We also found a strong expression of OPN mRNA in distal tubular cells in the kidneys of stone-forming rats [13]. OPN shows high-affinity binding to hydroxyapatite [17, 19], and appears to play a role in modulating the mineralization of calcifying tissues [13, 17, 19]. These and other reports suggest that OPN also plays a role in cell attachment and pathological calcium deposition such as that in urinary tract stones [13, 17, 19].

Bisphosphonate is one of the carbon-substituted pyrophosphate analogues which include potent inhibitors of bone resorption. It is very effective when administered to patients with accelerated bone absorption, such as in hypercalcemia induced by malignant tumor or osteoporosis [8, 14]. Urinary stones are very similar to bone diseases such as osteoporosis and bone metabolism in terms of the pathogenesis. The components of both urinary stones and bone include calcium, phosphate, osteopontin and collagen. Both diseases are

related to hormones such as parathyroid hormone and vitamin D_3, long bed rest and acidosis. Bisphosphonates have a strong affinity for calcium, with which they can form both soluble and insoluble complexes and aggregates [8]. The physicochemical effects of bisphosphonates are very similar to those of pyrophosphate. Thus, they inhibit the formation, delay the aggregation, and also slow down the dissolution of calcium crystals. Bisphosphonate is expected to show preventive effects against urolithiasis [6, 21].

Bisphosphonates are classified by generation according to their structural differences. Etidronate is an old generation bisphosphonate compound which has been used to cure osteoporosis [21]. It decreases calcium oxalate crystalluria in vivo and inhibits the crystallization and adhesion of calcium phosphate crystals to cultured cells in vitro [6, 21]. However, etidronate can not be used to treat urinary stones because the dosages required to prevent stone formation cause disorders in skeletal turnover and bone mineralization [2]. Subsequently, novel bisphosphonates classified as new generation have been synthesized. They are pharmacologically 100–1,000-fold more potent than etidronate in terms of bone antiresorptive activity, and are clinically applied to tumor-induced hypercalcemia and osteoporosis. Furthermore, they may be used against arteriosclerosis in the future [24]. Alendronate and incadronate are new generation bisphosphonate compounds.

The Madin-Darby canine kidney (MDCK) cell line, derived from a normal, adult male cocker spaniel kidney, expresses many characteristics of renal distal tubular cells in culture [5]. MDCK cells produce calcium phosphate microliths at the basolateral surface both in vitro [10, 16] and in the ex vivo subcutis of nude mice [23]. Naito et al. [16] established a model of calcium phosphate microlith formation using MDCK cells in three-dimensional soft agar culture and confirmed the formation of visible dark colonies containing calcium phosphate microliths, and clear colonies without microliths.

Here, we investigated the inhibitory effect of new generation bisphosphonates (alendronate and incadronate) on calcium phosphate microlith formation, as well as on the expression of osteopontin in MDCK cells cultured in three-dimensional soft agar.

Materials and methods

Cell culture

MDCK cells obtained from the Laboratory Products Division of Dainippon Pharmaceutical (Osaka, Japan) were passaged approximately 40 times and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 20 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Rockville, Md.). The cells were normally seeded at a density of 10^5 cells per 60 mm tissue culture dish (Nagle Nunc, Naperville, Ill.) at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were grown as monolayers in a plastic culture dish, and at confluence they formed domes occasionally containing calcium phosphate microliths [10, 16]. For three-dimensional soft agar culture, 100 cells were embedded in 2 ml 0.33% soft agar medium on a 2 ml 0.5% agar basal layer (feeding layer) in 60 mm plastic dishes for 42 days (Fig. 1). In detail, 2 ml of aseptic 0.5% agar was dissolved at 42°C in DMEM for the basal layer and placed in a 60 mm plastic dish. After the basal layer hardened at room temperature, 100 MDCK cells were added to 2 ml of 0.33% soft agar in DMEM dissolved at 42°C and put on the basal layer at room temperature. Culture medium (1 ml) was added every 7 days for feeding and to prevent the drying of the surface of the soft agar layer.

Light microscopy

MDCK cells cultured in three-dimensional soft agar culture for 42 days formed two types of colonies, dark and clear, as observed by phase contrast microscopy. Colonies were harvested and immediately fixed in 10% formalin for 24 h, embedded in paraffin, and cut into 4-μm thick sections. The paraffin sections were stained with hematoxylin and eosin for morphological examination and by von Kossa staining to detect microliths and calcification. Colony forming efficiency in three-dimensional soft agar culture was measured as the number of visible colonies of over 1 mm diameter relative to the number of initial cellular units at 42 days of culture. Colony efficiency (%) = visible colonies formed/number of seeded cells×100.

The inhibitory effect of bisphosphonate on microlith formation was evaluated as the number of dark colonies relative to the total number of colonies (dark plus clear colonies) at 42 days of culture, because dark colonies contained microliths but clear colonies do not. Dark colony ratio (%) = number of dark colonies/number of total colonies×100.

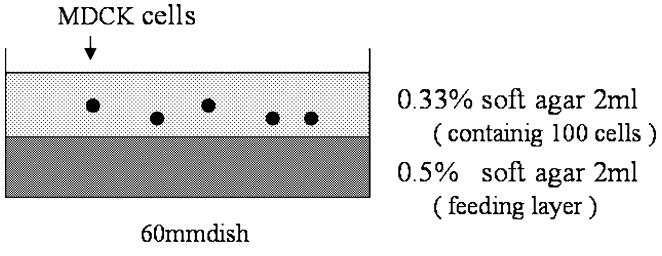

Fig. 1 Cross sectional image of three-dimensional soft agar culture. One hundred cells were embedded in 2 ml 0.33% soft agar medium (culture layer) on 2 ml 0.5% agar-basal layer (feeding layer) in 60 mm plastic dishes

Administration of bisphosphonates

Alendronate was obtained from Merck Research Laboratories (West Point, Pa.), and incadronate was obtained from Yamanouchi Pharmaceutical (Tokyo, Japan). Powdered bisphosphonates were dissolved in DMEM containing 10% FBS supplemented with 1% penicillin and streptomycin to final concentrations of 10^{-11} M, 10^{-9} M, 10^{-7} M and 10^{-5} M. Purified MDCK cells were divided into nine groups of ten dishes as follows: a control group with only DMEM, four groups in DMEM containing 10^{-11} M, 10^{-9} M, 10^{-7} M and 10^{-5} M alendronate and four groups in DMEM containing 10^{-11} M, 10^{-9} M, 10^{-7} M and 10^{-5} M incadronate. Each solution (1 ml) was added to the dish surfaces once per week (0, 7, 14, 21, 28, 35 days). The concentrations of bisphosphonate used in this study were selected on the basis of concentrations of alendronate bisphosphonates used in experiments with neonatal mice calvaria investigating the inhibitory effects on osteoclastic bone resorption [1], and studies with rabbit osteoclasts investigating cytotoxic effects in vitro [9].

Von Kossa staining

We detected calcium phosphate microliths using the von Kossa procedure [15]. Sections were rinsed twice between the following steps with distilled water for 5 min. The sections were deparaffinized, immersed in 5% silver nitrate, illuminated for 60 min, and then immersed in 5% sodium thiosulfate for 3 min before counterstaining with Kernechtrot solution for 5 min.

Immunohistochemical staining

We visualized OPN expression by immunohistochemically staining paraffin sections that were blocked with 3% H_2O_2 in methanol for 30 min, and washed with 0.01 M PBS. Specific binding was blocked by incubating the sections at room temperature for 30 min with 1% normal rabbit serum. The sections were then incubated with rabbit polyclonal anti-human OPN for 30 min at room temperature. Antibody binding was demonstrated using a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.) for paraffin sections. Signals were detected using the New Fuchsin Substrate System (DAKO, Carpinteria, Calif.) and DAB chromogen, respectively.

Statistical analysis

All data are expressed as means ± standard deviation (SD). The Student's *t*-test was used to detect significant differences between groups. A P value below 0.05 indicated a significant difference.

Results

MDCK cells cultured in three-dimensional soft agar

Cultured cells grew gradually and formed visible colonies with a diameter of approximately 0.5 mm at 14 days (Fig. 2), and reached approximately 2–3 mm in diameter at 42 days. Phase contrast microscopy revealed two types of colonies, dark (Fig. 3A) and clear colonies (Fig. 3B), at 42 days of culture. Almost all colonies were easily distinguished as either clear or dark on the 14th day by phase contact microscopy. Staining with H-E revealed that pseudocysts were abundant in the dark (Fig. 3C), but not in the clear (Fig. 3D) colonies. Microliths were observed within pseudocysts formed by a single MDCK cell layer in dark colonies at 42 days of culture, although no microliths were observed in clear colonies, as in Naito's report [16].

Colony efficiency and dark colony ratio in three-dimensional soft agar culture

The colony efficiency of the control group (without bisphosphonate) was 26.8 ± 2.8%. There was no significant difference in colony efficiency between control and three alendronate groups (concentrations: 10^{-11} M, 10^{-9} M and 10^{-7} M: 27.4 ± 3.6%, 27.1 ± 3.9% and 26.5 ± 3.3%, respectively). No colonies formed in the group exposed to 10^{-5} M alendronate. There was no significant difference in colony efficiency between the control and three incadronate groups (concentrations: 10^{-11} M, 10^{-9} M and 10^{-7} M: 29.0 ± 5.3%, 24.2 ± 7.0% and 27.7 ± 4.7%, respectively). No colonies formed in the group exposed to 10^{-5} M incadronate.

The dark colony ratio of the control group was 9.4 ± 2.8%. Although the 10^{-11} M alendronate group did not significantly differ, the dark colony ratio of the 10^{-9}

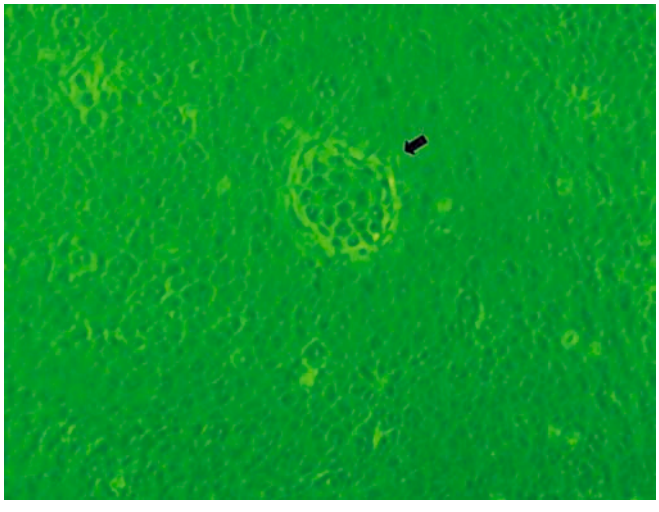

Fig. 2 The dome formed by MDCK cells at confluence (*arrow*) observed by phase contrast photomicrograph (×100)

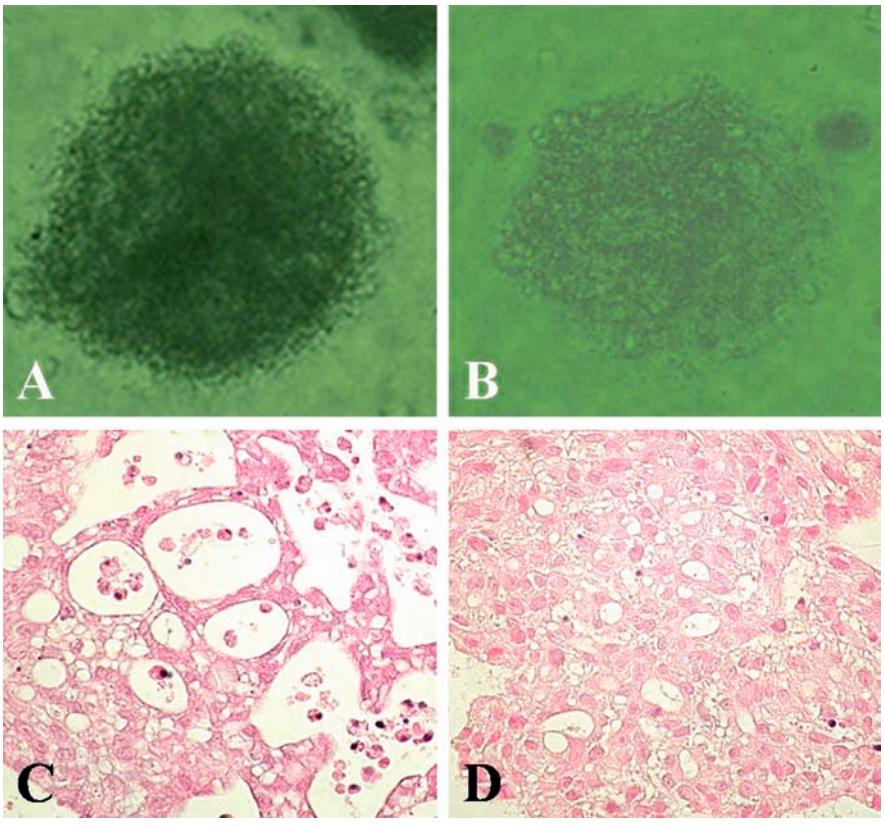

Fig. 3 Colony originating from single MDCK cell after 42 days in three-dimensional soft agar culture. Phase contrast microscopy revealed two types of colonies, **A** dark and **B** clear colonies (×40). H-E staining revealed that pseudocysts were abundant in dark (**C**), but not in clear (**D**) colonies (×200)

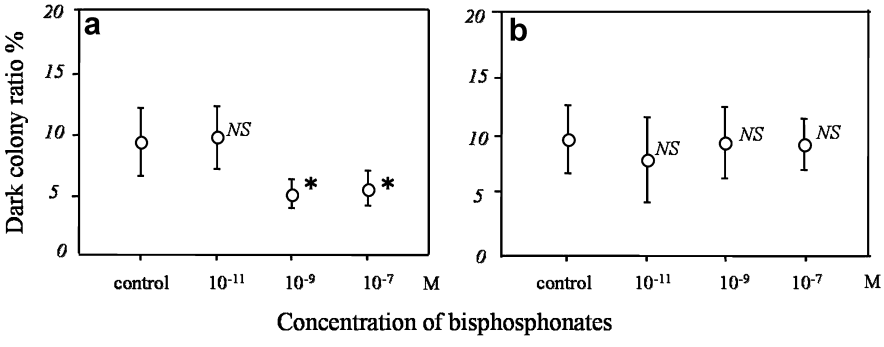

Fig. 4 Dark colony ratio in three-dimensional soft agar culture. **a** alendronate group; **b** incadronate group. * $P < 0.01$: significantly different from the control group. NS, not significantly different from control group

and 10^{-7} M alendronate groups was decreased compared with control ($P < 0.01$) (10^{-11}, 10^{-9} and 10^{-7}M: 9.8 ± 2.5, 5.2 ± 1.1 and 5.6 ± 1.5 %, respectively) (Fig. 4a). However, the dark colony ratio between the control and the three incadronate groups did not differ significantly (10^{-11}, 10^{-9} and 10^{-7} M: 7.8 ± 3.5, 9.2 ± 2.9 and 9.4 ± 2.1%, respectively) (Fig. 4b).

OPN expression in cultured cells

OPN was expressed in MDCK cells of all groups at 42 days of culture. Less OPN was expressed in the 10^{-7} M alendronate group than in the control group, although the amount of OPN expressed by the 10^{-7} M incadronate group was similar to that in the controls (Fig. 5).

Discussion

Urinary stone formation is the result of a cascade of events including nucleation, growth, aggregation and crystallization, but whether stones are generated at the luminal or basolateral side of renal convoluted tubules remains unknown. Kageyama et al. demonstrated that MDCK cells produced microliths (calcium phosphate) at the basolateral side in monolayer cultures [10]. We confirmed that MDCK cells transplanted subcutaneously into nude mice formed follicles inside the basolateral side and calcium phosphate microliths ex vivo [23]. MDCK cells transport calcium and phosphate ions from the apical to the basal side of the cell layer [18]. Naito et al. [16] established three-dimensional soft agar gel culture using MDCK cells and confirmed the

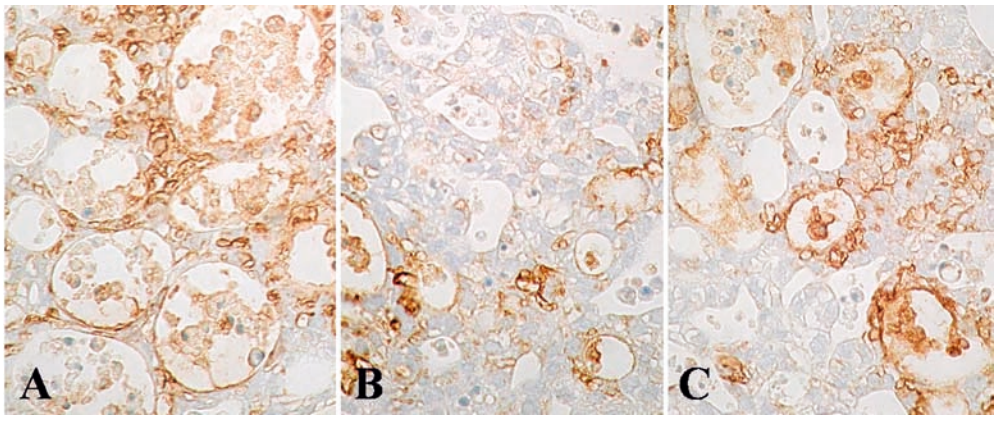

Fig. 5 Immunohistochemical staining using osteopontin polyclonal antibody (*brown*) in **A** the control group, **B** 10^{-7} M alendronate group, and **C** 10^{-7} M incadronate group (×200)

formation of visible dark colonies containing calcium phosphate microliths, and clear colonies without microliths. We used this experimental model because three-dimensional gel culture is in vitro, whereas finished colonies are more similar to the environment in vivo.

Bisphosphonates have a phosphorate-carbonate-phosphorate (P-C-P) structure in which carbon is substituted for oxygen [7]. They develope as stable analogues of inorganic pyrophosphoric acid with different structures. Alendronate decreases urinary calcium excretion and supersaturation in the hypercalciuric rat [4], and prevents hypercalciuria and the crystallization of calcium salts that occur during strict bed-rest, causing urolithiasis [22]. On the other hand, the effect of incadronate on the formation of urinary stones has not yet been investigated.

In the present study, we examined the inhibitory effect of alendronate and incadronate on calcium phosphate microlith formation using three-dimensional soft agar gel culture. MDCK cells formed no colonies on administration of 10^{-5} M alendronate or 10^{-5} M incadronate. Therefore 10^{-5} M alendronate or incadronate caused cell cytotoxicity and were thought to represent an overdose. The administration of either alendronate or incadronate in 10^{-11}, 10^{-9} and 10^{-7} M groups did not change colony efficiency. These results show that there was less cytotoxicity in bisphosphonate at these concentrations. The decline of the dark colony ratio showed an inhibitory effect on stone formation, because microliths were observed only in dark colonies. Alendronate at 10^{-9} M and 10^{-7} M significantly decreased the dark colony ratio, whereas no incadronate concentrations had any effect. This suggests that alendronate inhibits calcium stone formation. The P-C-P structure of bisphosphonates allows considerable variation, either by changing the two lateral chains on the carbon atom, or by esterifying the phosphate groups. Thus, each bisphosphonate has unique physicochemical and biological characteristics. New generation bisphosphonates are categorized as second or third generation according to structure. Second generation bisphosphonate has nitrogen-containing structure, and third generation bisphosphonate has a circular structure of the R^2 side chain. Alendronate belongs to the second generation and incadronate belongs to the third generation. A difference in the lateral chain structure might explain the inhibitory effect on microlith formation found in the present study.

Urinary stones contain 1–5% protein, and several investigators have discussed the importance of proteins in stone formation [3]. We subsequently identified OPN from a urinary stone matrix and detected its expression in the renal distal tubular cells of stone-forming rats [12, 13]. The production of OPN is gradually increased in response to renal epithelial cell exposure to oxalate and calcium oxalate crystals and OPN plays a significant role in calcium nephrolithiasis [11]. We recently reported that alendronate inhibited the elevation of OPN protein in the distal tubular cells of hypercalcemic rats undergoing treatment with PTHrP [26]. In this study, we observed OPN expression by immunohistochemical staining in dark colonies formed by MDCK cells. Less OPN was expressed in the alendronate than in the control and incadronate groups. Greater expression of OPN is observed at stone formation. The decline of the OPN expression suggests repression of stone formation. This in turn suggests that alendronate inhibited stone formation more effectively than incadronate. However, it is unclear whether alendronate controlled the expression of OPN directly or alendronate controlled microlith formation, which in turn decreased OPN expression.

In conclusion, the present study demonstrated that alendronate reduced the dark colony ratio and osteopontin expression in soft agar gel cultures, whereas incadronate did not. Thus, alendronate appears to inhibit calcium stone formation, suggesting that it will help to prevent urolithiasis.

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