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Effects of citrate on renal stone formation and osteopontin expression in a rat urolithiasis model

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Abstract Previous studies have described the inhibitory effects of citrate on calcium oxalate crystallization in place of crystal growth, but the effects of citrate on matrix proteins of stones has not been studied in vivo. To examine the effect of citrate on the matrix, we investigated the effect of citrate on osteopontin (OPN) expression, which we had previously identified as an important stone matrix protein. Control rats were treated with saline while rats of the stone group were treated with ethylene glycol (EG) and vitamin D_3, and the citrate groups (low-dose and high-dose groups) were treated with a citrate reagent compound of sodium citrate and potassium citrate, in addition to EG and vitamin D_3. The rate of renal stone formation was lower in the citrate groups than in the stone group. This was associated with a low expression of OPN mRNA in citrate-treated rats relative to that in the stone group. Citrate was effective in preventing calcium oxalate stone formation and reduced OPN expression in rats. Our results suggest that citrate prevents renal stone formation by acting against not only the crystal aggregation and growth of calcium oxalate but also OPN expression.

Key words Calcium oxalate · Urolithiasis · Citrate · Osteopontin · Ethylene glycol

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Introduction

Calcium oxalate calculus of the kidney is a common clinical problem; population studies have shown that 1 in 1000 people pass a calcium oxalate calculus each year and that one in three cases of symptomatic renal calculi necessitates admission to a hospital [10, 18]. There is as yet no effective treatment for idiopathic oxalate calculi, though positive effects of thiazide for hypercalciuria and alkali citrate for hypocitraturia have been reported [18, 19].

Urinary stones contain 1–5% protein, and several studies have discussed the importance of proteins in stone formation [3]. We previously cloned and sequenced the cDNA encoding osteopontin (OPN), an important soluble stone protein component of calcium oxalate stone proteins extracted with 0.1 M EDTA [11]. We found a strong expression of OPN mRNA by distal tubular cells in the kidneys of stone-forming rats [12]. Citrate has received renewed interest as an important factor in the prevention of calcium urolithiasis. Oral potassium citrate therapy has also been widely accepted for the prevention of hypocitraturic calcium nephrolithiasis [1, 7]. A number of in vitro studies have suggested that the effect of citrate might be exerted both by direct actions on the formation and aggregation [13, 24] of calcium oxalate, and by enhancing the inhibitory effects of urinary macromolecules [9]. The effect of citrate on matrix proteins of stones has not yet been elucidated. Here, we investigated the effects of citrate on OPN expression in addition to its effect on the formation of calcium oxalate renal stones induced by ethylene glycol (EG) and vitamin D_3 in rats.

Materials and Methods

Animals

All experimental procedures were performed in accordance with protocols approved by the Animal Care Committee of the Faculty

of Medicine, Nagoya City University. In the in vivo study, we adopted the rat stone-forming model reported by Okada et al. [17] with a minor modification. We purchased 7-week-old male Wistar rats, approximately 240 g, from Charles River Japan (Yokohama, Japan). A standard diet for rats (MEQ; including Ca 1.01 g, P 0.78 g, Mg 0.21 g, Na 0.23 g/100 g, from Oriental Yeast, Tokyo, Japan) was used. To induce calcium oxalate deposits, rats were treated by gastric gavage: (1) 0.5 μg vitamin D_3 ($1\alpha(OH)D_3$, alfacalcidol) (Chugai Pharmaceutical, Tokyo) every other day, and (2) 0.12 ml of 5% EG (Wako, Tokyo) in 1.0 ml of water daily in two doses. A citrate reagent (Uralyt, Nippon Chemiphar, Tokyo), a compound of sodium citrate and potassium citrate (Na: 4.5 mEq/g, K: 4.5 mEq/g), was used. The citrate reagent was administered twice daily at 0.5 g/kg per day (low-dose group) or 2.0 g/kg per day (high-dose group) in 1.0 ml of water daily (at the times when EG and vitamin D_3 were not administered) through a stomach tube. Saline was administered in control rats.

Experimental protocol

After 1 week of acclimatization, rats were divided into four groups, each consisting of 20 rats: (1) control rats treated with saline (control group), (2) rats administered EG and vitamin D_3 (stone group), (3) rats administered EG, vitamin D_3, and citrate (0.5 g/kg per day citrate) (low-dose group), and (4) rats administered EG, Vitamin D_3, and citrate (2.0 g/kg per day) (high-dose group). Citrate reagent was administered at about five (low-dose group) or 20 (high-dose group) times the equivalent of the human daily dose per unit of body weight in the experimental rat model. The rats were weighed weekly. Pooled 24-h urine samples from each group were collected weekly with the use of metabolic cages, and the urine samples for the measurement of oxalate were collected in cups containing hydrochloric acid (HCl). Following collection of blood samples from the inferior vena cava, five rats from each group were killed under ether anesthesia at 0, 7, 14, and 28 days after the initiation of treatment, and both kidneys were excised. One kidney was used for histological examination while the other was used for RNA extraction. Samples of urine were obtained from each rat two days before death by individually housing the animal in a metabolic cage for 24 h.

Measurement of blood and urinary variables

Serum calcium, phosphorus, and blood urea nitrogen (BUN) and urinary calcium and citrate levels were determined with an automatic analyzer (Model 705, Hitachi, Tokyo) after centrifugation. Oxalate was measured by Fraser and Campbell's method [8]. Briefly, 20 ml of urine was centrifuged to remove solid matter, then adjusted to pH = 4.5 with NH_4OH solution. To this, we added 0.4 ml of 2 mg/ml $Na_2C_2O_4$ solution and 0.4 ml of 100 mg/ml $CaCl_2$ solution. The mixture was heated to 100 °C, then cooled to room temperature, and its pH was readjusted to 4.5 with a solution of NH_4OH or H_2SO_4. The mixture was then centrifuged and the precipitate was washed three times with a 6-ml saturated solution of CaC_2O_4 in water, and dissolved in 1 ml of 1N H_2SO_4 by heating at 60 °C for 5 min. Subsequently, 4 ml of distilled water was added, and then the solution was further diluted 25 times with water and its calcium content was determined with an atomic absorption spectrophotometer. Serum and urine samples were stored at −70 °C until analyzed.

Histological examination of the kidney

Excised kidney tissue samples were fixed with 4% paraformaldehyde in 0.1 *M* of phosphate buffer (pH 7.0), dehydrated in ethanol, and embedded in paraffin under RNase-free conditions. Serial sections (4 μm thick) were cut. For the evaluation of calcium deposits, sections were stained by the method described by Pizzolato [20] to demonstrate calcium oxalate and with von Kossa's method to demonstrate phosphate calcium. Though the results of these two staining methods were always consistent in the kidneys of this model, Pizzolato's method [20] yielded the clearest results.

Number of calcium oxalate deposits

Excised kidney tissue samples were cut sagittally, and calcium oxalate was detected by Pizzolato's method [20]. Deparaffinized and hydrated slides were placed at a 20-cm distance from a 60 W lamp for 30 min and flooded with a 1:1 mixture of 30% H_2O_2 and 5% $AgNO_3$. After this illumination, the sections were lightly counterstained in a 0.05% safranin O solution for 1 min and washed briefly, and then they were dehydrated and mounted. The total number of positive signals was divided by the area of the specimen and then normalized. The data presented are the number of detected calcium oxalate deposits per square centimeter (cut area).

Probe preparation for in situ hybridization

A 984-bp fragment of mouse 2ar (osteopontin) complementary cDNA was subcloned into Bluescript pKS(−) plasmid [16]. The plasmid was then linearized with *Eco*RI and transcribed with T3 RNA polymerase to generate an antisense cRNA probe. The plasmid was then linearized with *Xho*I and transcribed with T7 RNA polymerase to generate a sense cRNA probe. The specificity of these probes was confirmed by Northern blotting.

RNA extraction and Northern blotting

Tissue samples for Northern hybridization were frozen with liquid nitrogen, and total RNAs were extracted by the method described by Chirgwin et al. [4] from kidney tissues harvested from rats killed on days 7, 14, and 28. For Northern blotting, 20 μg total RNA was fractionated on a formaldehyde-agarose gel system and transferred to a Hybond N^+ nylon membrane (Amersham, Buckinghamshire, UK). The membranes were prehybridized and then hybridized with the [^{32}P]CTP-labeled probes, according to the instructions provided by the manufacturer. After hybridization, the membranes were washed and signals were measured by autoradiography. Equal loading of RNA was confirmed by the staining of 28S or 18S RNA bands with ethidium bromide. The relative mobilities of 18S (2.2 kb) and 28 S (4.7 kb) ribosomal RNAs are shown as size markers.

In situ hybridization

Details of the in situ hybridization technique used here have been described previously [16]. Digoxigenin-uridine triphosphate (UTP)-labeled single-strand RNA probes were prepared for hybridization using a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the protocol recommended by the manufacturer. Hybridization of OPN mRNA was performed at 50° for 16 h, and signals were detected with a nucleic acid detection kit (Boehringer Mannheim). The controls included: (1) hybridization with sense (mRNA) probes, (2) RNase treatment before hybridization, and (3) the use of antisense RNA or the removal of anti-digoxigenin antibody. No positive signals were observed under any of these three conditions.

Immunohistochemical staining

Immunohistochemical staining was carried out using paraffin sections, adjacent to those used for in situ hybridization and routine staining. MPIIIB10(1) (Developmental Studies Hybridoma Bank, Iowa City, Iowa), which recognizes osteopontin, was used as the primary antibody. Deparaffinized sections were incubated in 0.3% H_2O_2 in methanol for 30 min, followed by washing in 0.01 *M* phosphate-buffered saline (PBS). To block nonspecific binding, all sections were treated with 1% normal rabbit serum for 30 min at room temperature. The slides were then incubated with MPIIIB10(1) for 18 h at 4 °C. Antimouse IgG antiserum was used as the secondary antibody. The binding of the secondary antibody was demonstrated with an LSAB2 kit, AP, Rat (Dako, Glostrup, Denmark) for paraffin sections, according to the instructions

provided by the manufacturer. Signals were respectively detected with the New Fuchsin Substrate System (Dako) and a DAB chromogen. As negative controls, sections were incubated with nonimmune mouse serum instead of the primary antibodies, or with PBS, instead of the secondary antibody.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences between groups were examined for statistical significance using the Student's *t*-test. A P value < 0.05 denoted the presence of a significant statistical difference.

Results

Serum and urinary data

Blood and urine samples were collected at 7, 14, and 28 days ($n = 5$ at each day from each group) after the commencement of stone-formation therapy with or without citrate. At day 7 after initiation of therapy (EG and vitamin D_3), the 24-h urinary oxalate excretion in the stone group was more than three times that in the control rats (0.88 ± 0.41 vs 0.26 ± 0.11 mg/day, respectively). These results suggested that administration of EG and vitamin D_3 was effective in providing conditions favorable to the formation of oxalate deposits. Citrate treatment did not reduce urinary oxalate excretion compared to the stone group (Fig. 1). Urinary oxalate excretion had a tendency to increase gradually, but was not significantly different among days 7, 14, and 28 in the four groups. At 28 days, the concentration of serum BUN was not different among the four groups. Similarly, there were no significant differences in serum calcium and phosphorus levels between the stone group and both citrate groups. The 24-h urinary calcium excretion was not different between the stone group and citrate groups and urinary citrate excretion values in the citrate groups were higher than those in the control group and stone group, and dependent on the citrate dose (Table 1).

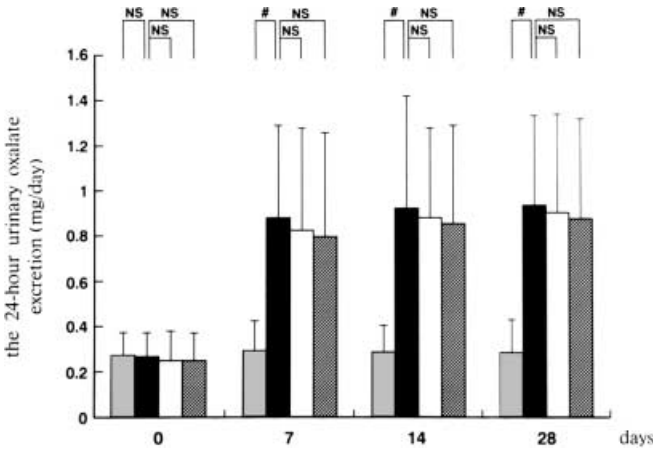

Fig. 1 The 24-h urinary oxalate excretion at different intervals after commencement of therapy in the control group (*gray bars*), stone group (*solid bars*), low-dose group (*open bars*), and high-dose group (*hatched bars*). Stone group; $P < 0.05$: significantly different from the control group. Low-dose group and high-dose group. *NS* not significant from the stone group

Histological examination

Pizzolato's staining method [20] clearly demonstrated the presence of calcium oxalate deposits (Fig. 2a) in the kidneys of the stone group and citrate groups after 28 days of treatment. On the other hand, some of the deposits were stained by von Kossa's method (data not shown), although such staining was not clearer in most preparations than Pizzolato's method. No deposits were detected at 7, 14, and 28 days in the control rats. At 7 days, calcium deposits were detected in one of five rats of the stone and citrate groups. However, at 14 and 28 days, calcium deposits were detected in all five rats of the stone and citrate groups. A linear increase in the number of deposits was observed after days 7, 14, and 28 in the stone and citrate groups. However, quantitative analysis of the density of calcium deposits showed a significantly lower density of deposits in the citrate-treated groups at days 14 and 28 compared to the stone group (Fig. 2b). The number of these deposits in the high-dose group was not, however, significantly different from that of the low-dose group.

Immunohistochemical studies

The OPN protein expression was observed in the kidneys of all four groups (Fig. 3a, c, e) after 28 days of

Table 1 Serum and urinary data in control and various treatment groups. Data are mean ± SD. Samples were collected at 28 days after commencement of treatment

	Control group[b]	Stone group[c]	Low-dose group[d]	High-dose group[e]
Urinary oxalate excretion (mg/day)[a]	0.2 ± 0.2	1.0 ± 0.5	0.9 ± 0.4	0.8 ± 0.4
BUN (mg/dl)	20.1 ± 2.1	26.4 ± 5.3	20.6 ± 1.3	26.1 ± 4.9
Serum calcium (mg/dl)	10.8 ± 0.5	12.5 ± 2.3	12.0 ± 1.0	11.9 ± 0.4
Serum phosphorus (mg/dl)	11.4 ± 1.4	11.8 ± 2.3	11.5 ± 1.7	10.9 ± 1.7
Urinary calcium excretion (mg/day)	2.2 ± 1.0	3.6 ± 1.8	4.1 ± 2.3	3.7 ± 2.2
Urinary citrate excretion (mg/day)	10.8 ± 4.0	15.5 ± 3.2	35.9 ± 7.2	48.5 ± 10.2

[a] Measurements performed after citrate therapy
[b] Control group: rats treated with saline
[c] Stone group: rats administered EG and vitamin D_3
[d] Low-dose group: rats administered EG, vitamin D_3, and citrate (0.5 g/kg per day citrate)
[e] High dose group: rats administered EG, Vitamin D_3, and citrate (2.0 g/kg per day)

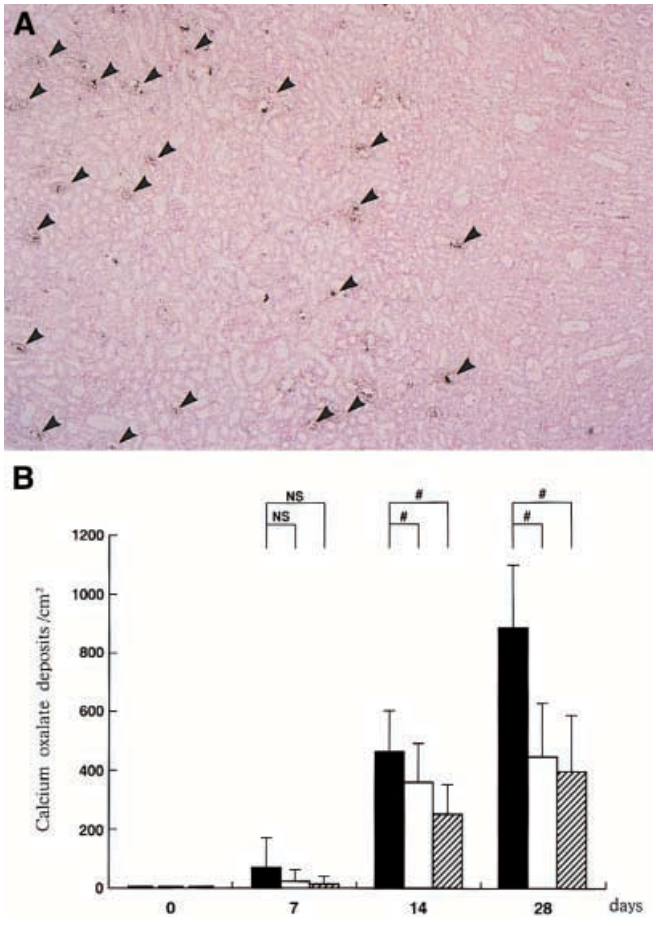

Fig. 2 **a** Microscopic appearance of rat kidneys. Sections stained by Pizzolato's method showing calcium oxalate deposits (*arrowheads*) in the kidneys of the stone group at 28th day of treatment. Magnification, ×40. **b** Density of calcium oxalate deposits in rat kidneys examined with Pizzolato's method in the stone group (*solid bars*), low-dose group (*open bars*), and high-dose group (*hatched bars*). Data represent the mean ± SD of the number of calcium oxalate deposits detected/cm^2 (cut area). Low-dose group and high-dose group; $P < 0.05$: significantly different from the stone group. *NS* not significant

treatment. The OPN proteins were found in both the distal and proximal convoluted tubules, the loop of Henle, and collecting ducts (mainly the medullary thick ascending limb of the loop of Henle), whereas no staining for the protein was detected in glomeruli in the renal cortex. In rats of the control group, only a weak OPN staining was noted (Fig. 3a). However, the expression was enhanced in the stone group (Fig. 3c). Citrate-treated rats tended to have lower OPN expression than the stone group (Fig. 3e).

In situ hybridization

In situ hybridization performed after 28 days of treatment demonstrated the expression of OPN mRNA in both the distal and proximal convoluted tubules, loop of Henle, and the collecting ducts (mainly the medullary thick ascending limb of Henle's loop); the glomeruli were negative (Fig. 3b, d, f). In kidneys from the control group, OPN mRNA was detected in a small proportion of the loops of Henle in the renal medulla (Fig. 3b). In contrast, the kidneys from the stone (Fig. 3d) and citrate (Fig. 3f) groups showed overexpression of OPN mRNA; however, the increase in the citrate groups was relatively weak compared to the stone group.

Northern blot analysis

After 28 days of treatment, examination of the gene expression in rat kidneys by Northern blot analysis using OPN cDNA as a probe revealed a faint signal of approximately 1.6 kb in control group rats (Fig. 4). In the stone group, the rat kidney OPN mRNA signal was clearly enhanced. The enhancement commenced at least 7 days after the start of treatment. The expression level of OPN mRNA in the citrate groups was enhanced, but was relatively weaker than in the stone group. Furthermore, the expression of OPN in the high-dose group was weaker than in the low-dose group.

Discussion

Citrate can affect the crystallization of calcium oxalate through several mechanisms. The formation of strong complexes with calcium citrate affects the ion-activity product of both calcium oxalate and calcium phosphate. Citrate forms complexes with calcium, thereby decreasing urinary saturation, and has been shown to inhibit the aggregation of calcium oxalate crystals [7, 13]. In addition, urinary alkalinization by potassium citrate increases the solubility of uric acid and thus prevents the salting out of calcium oxalate by urate. To our knowledge, there are no studies that have previously examined the effects of citrate on matrix proteins of renal stones. In the present study, we evaluated the effectiveness of citrate on stone formation and OPN expression in an in vivo rat model.

We administered low-dose EG and vitamin D_3 to rats by gastric gavage to induce calcium oxalate stones in the kidney. We chose this experimental model because the renal dysfunction thus produced is not severe, and the stones build up slowly and gradually. Gavage feeding ensures a more accurate dosage than adding the medicine to the animals' drinking water. We administered a higher dose of citrate to the rats than a clinical dose per weight for humans, considering their body surface. The composition of deposits induced by this method is thought to be calcium oxalate containing calcium phosphate, based on our observations of staining using Pizzolato's and von Kossa's methods. Our results showed that citrate administration prevented calcium deposit formation and OPN expression.

We previously reported OPN in the matrix of urinary calcium stones [12] and suggest OPN acts on calcium

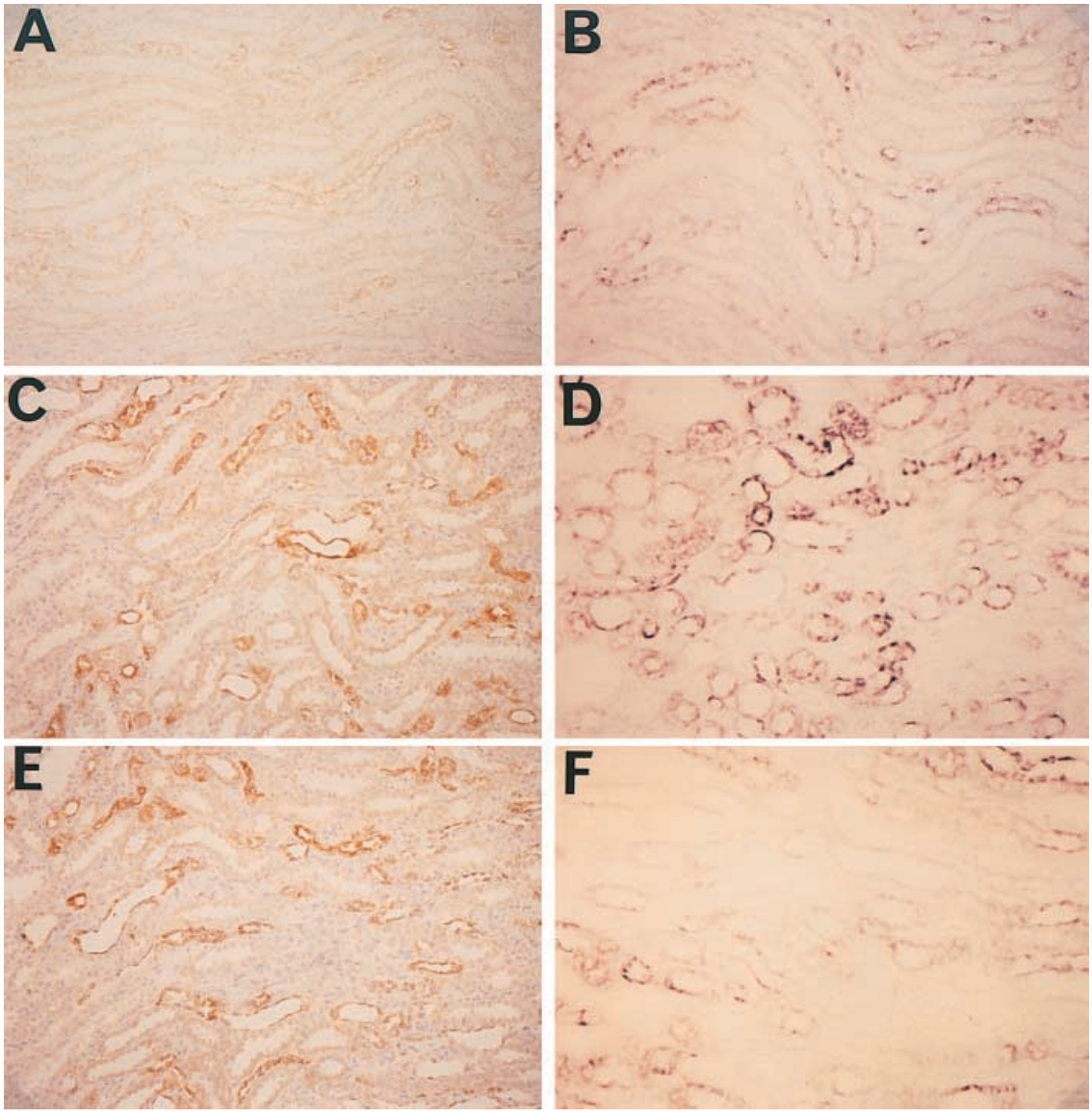

Fig. 3 Microscopic appearance of rat kidneys in the control group (**a**, **b**), stone group (**c**, **d**), and citrate group (high-dose group) (**e**, **f**) at 28 days of treatment. Immunohistochemical staining using OPN monoclonal antibody (*brown*) (**a**, **c**, **e** ×200). In situ hybridization analysis of OPN mRNA (**b**, **d**, **f** ×200)

stone formation [27]. Certain noncollagenous and plasma proteins, including OPN, sialoproteins, albumin, and α_2HS-glycoprotein [2] known to accumulate in bone and other mineralized tissues are also found in human renal stones. Of these, OPN is the major constituent of calcium oxalate-associated crystal ghosts observed in the nuclei, lamellae, and striations of organic matrix material in deposits within epithelial cells [15]. This suggests that during urolithiasis, the secretion of OPN and its subsequent incorporation into stone matrices may influence the nucleation and aggregative growth of stones [15]. Shiraga et al. [22] isolated an inhibitor of calcium oxalate crystal growth from human urine by monoclonal antibody immunoaffinity chromatography and named it uropontin. The N-terminal sequence from residues 1–44 of uropontin showed complete homology with human OPN. Furthermore, uropontin has an inhibitory effect on calcium oxalate crystal growth in vitro. OPN is also speculated to have an inhibitory effect on calcium oxalate crystallization in vitro, because it is abundant in acidic amino acids and binds firmly to hydroxyapatite. We extracted and identified OPN from renal stone proteins, and it will be important to determine the role of OPN in renal stone formation in vivo [11, 12]. Although uropontin is an inhibitor of calcium oxalate crystal growth in vitro, it may not play the same role in vivo. It has thus been hypothesized that in the specific matrix environment, uropontin undergoes a configurational change in its hairpin structure whereby it enhances crystallization [21].

A recent study suggested that increased nucleation of calcium oxalate monohydrate (COM) crystals in the nephron lumen and the ensuing crystal-cell interactions could up-regulate OPN gene expression and protein

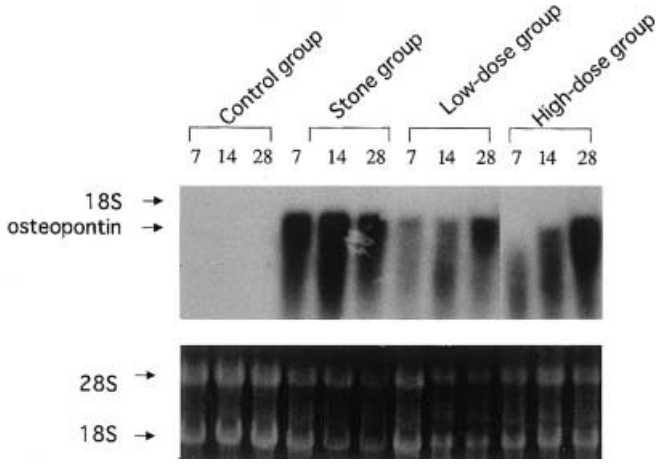

Fig. 4 Northern blot analysis of the expression level of OPN mRNA in the rat kidney of the control group, stone group, low-dose group, and high-dose group. The transcription of an approximately 1.6 kb mRNA probed for OPN (*top*) relative to the 28S and 18S ribosomal RNA bands (*bottom*) is shown. *Arrows* indicate 1.6 kb and 18S

secretion by renal tubular cells [14]. Enhanced OPN production by renal cells after interaction with COM crystals could have other important biological consequences, especially if OPN is released across the basolateral plasma membrane into the interstitium [5]. Urinary calcium oxalate crystals and crystal agglomerates are normally excreted, but in nephrolithiasis they are retained by tubular epithelial cells and shifted into the renal interstitium. This crystalline material induces an inflammatory response consisting of increases in the number of interstitial cells and the amount of the extracellular matrix [25]. In any case, OPN expression is enhanced in the process of stone formation, and examination of OPN expression is useful for confirming the process of stone formation and the efficiency of drugs used for the inhibition of stone formation. Immunohistochemical staining using the OPN antibody showed positive staining of the matrix of the urinary calcium stones in two distinct zones: a core area was stained with randomly aggregated OPN and peripheral layers ware stained in concentric circles [23].

Based on the above findings, we propose the following scheme on the role of osteopontin in the formation of kidney stones. The primary role of OPN appears to be facilitation of recovery after injury or infection, which generally causes an increase in its expression [6]. Inflammation causes expression of osteopontin as a chemotactic or haptotactic factor. Excessive concentration of oxalate or adhesion of calcium oxalate to renal epithelial cells, together with inflammation, enhances stone formation. It is conceivable that the immune response to inflammation enhances OPN expression. Thus, OPN can be viewed as a glue that facilitates the formation of calculi at sites of oxalate salt deposition; the basal membrane side of the renal tubules. Furthermore, in the growth of stones, OPN in urine attaches to stone nuclei and plays a role as glue. We reported that OPN excretion in urolithiasis patients was reduced, presumably because of the incorporation of OPN into urinary stones [26].

In this study, the expression of OPN in the stone group was already increased to a maximal level at 7 days after commencement of treatment, when deposits were just forming. This finding suggests that OPN expression is enhanced by the EG and vitamin D_3 administration, though deposit formation was not. The expression of OPN in citrate-treated rats was weak compared to the stone group, and increased little by little, though less than in the stone group. These findings suggest that inhibition of OPN expression is one of the mechanisms underlying citrate-induced inhibition of stone formation.

In conclusion, we demonstrated in the present study that administration of ethylene glycol and vitamin D_3 resulted in the formation of calcium oxalate deposits and increased OPN expression in rat kidneys. Citrate treatment decreased the formation of calcium oxalate deposits and OPN expression.

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