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The effect of takusha, a kampo medicine, on renal stone formation and osteopontin expression in a rat urolithiasis model

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Abstract Kampo medicine is a traditional Japanese therapeutic system which originated in China and was used to treat various diseases for hundreds of years. Kampo medicine had been also used for the cure and the prevention of urinary calculi for many years, but the effect and the mechanism of this use of kampo medicine are unclear. We examined the inhibitory effect of the kampo medicine takusha on the formation of calcium oxalate renal stones induced by ethylene glycol (EG) and vitamin D_3 in rats. We also investigated the effect of takusha on osteopontin (OPN) expression, which we previously identified as an important stone matrix protein. The control group rats were non-treated; the stone group rats were administered EG and vitamin D_3, and the takusha group was administered takusha in addition to EG and vitamin D_3. The rate of renal stone formation was lower in the takusha group than in the stone group; thus, the OPN expression in the takusha group was smaller than in the stone group. Takusha was effective in preventing oxalate calculi formation and OPN expression in rats. These findings suggest that takusha prevents stone formation including not only calcium oxalate aggregation but also proliferation.

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Introduction

Calcium oxalate calculi of the kidneys is a common clinical problem; population studies show that 1 in 1000 people pass a calcium oxalate calculus each year and that one in three cases of symptomatic calculi necessitates admission to hospital [1, 8]. There is as yet no effective treatment for idiopathic oxalate calculi, though a positive effect of thiazide for hypercalciuria and alkali citrate for hypocitraturia has been reported [17]. Kampo medicine is a traditional Japanese herbal therapeutic system that originated in China; the word kampo means "medicine from China". The kampo medicine chorei-to is used commonly to cure and to prevent recurrent calcium oxalate stone formation [12] not only in patients with hyperuricosuria, but also in patients with a normal urate excretion, but the effect and the pathway of action of chorei-to are unclear. Takusha, one of the components of chorei-to and whose herb origin is *Alisma orientale*, is cultivated in China. Takusha has been reported to be an in vitro and in vivo inhibitor of the crystallization of stone-forming calcium oxalate [11, 21]. In the present study, we examined the effect of Takusha on the formation of calcium oxalate renal stones induced by ethylene glycol (EG) and vitamin D_3 in rats.

Urinary stones contain 1%–5% protein, and many reports have suggested the importance of proteins in stone formation [3]. We recently cloned and sequenced the cDNA encoding osteopontin (OPN), an important soluble component of calcium oxalate stone proteins, extracted with 0.1 M EDTA [9]. We found a strong expression of OPN mRNA by distal tubular cells in the kidneys of stone-forming rats [10]. Here, we investigated the effect of takusha on OPN expression in addition to stone formation.

Materials and methods

Experimental animals and histology

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. [16] with minor modification. We purchased 7-week-old male Wistar rats, approximately 240 g, from Charles River Japan (Yokohama, Japan). Normal 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 given through a stomach tube (1) 0.5 μg vitamin D_3 ($1\alpha(OH)D_3$, alfacalcidol) (Chugai Pharmaceutical, Tokyo, Japan) every other day and (2) 0.12 ml of 5% EG (Wako, Tokyo, Japan) in 1.0 ml of water daily in two doses. Takusha was administered twice daily at 800 mg/kg per day, (at the times when EG and vitamin D_3 were not administered) through a stomach tube. This dose is about five times as large as a general clinical dose. Control rats were administered saline. After 1 week of acclimatization, rats were divided into three groups, each of 20 rats (1) control rats receiving saline (control group), (2) rats administered EG and vitamin D_3 (stone group), (3) rats administered EG, vitamin D_3 and takusha (takusha group). Takusha was given at five times the equivalent human daily dose per unit body weight in the experimental rat model.

The rats were weighed weekly. Pooled 24-hour 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). After blood samples were collected from the inferior vena cava, five animals from each group were killed under ether anesthesia at 0, 7, 14, and 28 days after the initiation of drug administration, and both kidneys were excised. One kidney was used for histologic examination and the other was used for RNA extraction. Samples of urine were obtained from each rat 2 days before death by individually housing the animal in a metabolic cage for 24 hours.

Serum calcium, phosphorus, creatinine and blood urea nitrogen (BUN) and urinary calcium levels were determined with an automatic analyzer (Model 705, Hitachi, Tokyo, Japan) after centrifugation. Oxalate was measured by the method of Fraser and Campbell [7]. Briefly, 20 ml of urine centrifuged to remove solid matter was adjusted to pH 4.5 with NH_4OH solution. To this, 0.4 ml of 2 mg/ml $Na_2C_2O_4$ solution and 0.4 ml of 100 mg/ml $CaCl_2$ solution were added. This mixture was heated to 100°C, then cooled at 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. The precipitate was washed three times with 6 ml saturated solution of CaC_2O_4 in water, and dissolved in 1 ml of 1 N 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.

Excised kidney tissue samples for histologic examination 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 using Pizzolato's method [18] 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 gave the clearest results.

The number of calcium oxalate deposits

Excised kidney tissue samples were cut sagittally, and calcium oxalate was detected by Pizzolato's method [18]. Deparaffinized and hydrated slides were placed at a 20-cm distance from a 60 W lamp for 30 minutes 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 minute 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 numbers of detected calculi/cm^2 (cut area).

Probe preparation for in situ hybridization

A 984-bp fragment of mouse 2ar (osteopontin) complementary cDNA was subcloned into Bluescript pKS(-) plasmid [15]. The plasmid was then linearized with EcoR I and transcribed with T3 RNA polymerase to generate an antisense cRNA probe. Then, the plasmid was 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 of Chirgwin et al. [4] from kidney tissues from animals killed on days 7, 14, and day 28. For Northern blotting, 20 μg total RNA was fractionated on a formaldehyde/agarose gel system and transferred to a Hybond N^+ nylon membranes (Amersham, UK). The membranes were prehybridized and then hybridized with the [^{32}p]CTP-labeled probes, according to the manufacturer's instructions. After hybridization, the membranes were washed and signals were measured by autoradiography. The same membrane was used for all probes. The equal loading of RNA was confirmed by either staining of the 28S and 18S RNA bands with ethidium bromide. The relative mobilities of the 18S (2.2 kb) and 28S (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 [15]. Digoxigenin-uridine triphosphate (UTP)-labeled single-strand RNA probes were prepared for hybridization using a DIG RNA labeling kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Hybridization of OPN mRNA was performed at 50°C for 16 hours, and the 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) use of antisense RNA or removal of antidigoxigenin antibody. No positive signals were observed under any of these three conditions.

Immunohistochemical staining

Immunohistochemical staining was carried out using the adjacent paraffin sections, which were used for in situ hybridization and routine staining. MPIIIB10(1) (Developmental Studies Hybridoma Bank, Iowa) was used as the primary antibody. MPIIIB10(1) recognizes osteopontin. Deparaffinized sections were incubated in 0.3% H_2O_2 in methanol for 30 minutes, 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 minutes at room temperature. The slides were then incubated with MPIIIB10(1) for 18 hours at 4°C. Anti-mouse 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 manufacturer's instructions. 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

The data are expressed as mean ± standard deviation (SD), and Student's *t*-test for paired values was applied to assess the significance of differences.

Results

The in vivo animal experiment

The takusha-treated rats were compared with the control group and stone group. Though the serum creatinine and BUN levels were slightly higher in both the stone group and takusha group compared with the control group after 28 days of treatment, the renal failure was not severe. Throughout the experiment, the 24-hour urinary excretion of oxalate was significantly higher in both the stone group and takusha group compared with that in the control group (Table 1). Regarding the serum calcium and urinary oxalate excretions, there were no significant differences between the stone group and the takusha group. The 24-hour urinary excretions of calcium in both the stone group and takusha group were significantly higher than in the control group. No renal stone was observed in the urinary tract of any animal at death.

Calcium oxalate deposits

Pizzolato's method clearly revealed calcium oxalate deposits in the kidneys of the stone group and takusha group (Fig. 1B, C). No deposits were detected in the kidneys of the control group rats (Fig. 1A). Some of the deposits were stained partially by von Kossa's method (data not shown), but not clearly. Microscopic findings in the rat kidneys revealed the apparent prevention of calcium oxalate deposition in the takusha group compared with the stone group. The numbers of calcium oxalate deposits are shown in Fig. 2. The takusha group exhibited a significant decrease in tissue calcium oxalate deposits in the kidney compared with the stone group.

Osteopontin expression

OPN protein expression was observed in the kidneys of all three groups (Fig. 1D–F). The OPN proteins were found in both the distal and proximal convoluted tubules, the loops of Henle and collecting ducts (mainly the medullary thick ascending limb of Henle's loop), whereas the glomeruli in the renal cortex were negative. In the control group, the OPN staining was only weak (Fig. 1D).

The expression sites of OPN mRNA demonstrated by in situ hybridization were both the distal and proximal convoluted tubules, the loops of Henle and collecting ducts (mainly the medullary thick ascending limb of Henle's loop), whereas the glomeruli were negative (Fig. 1G–I). In the kidneys from the control group, OPN mRNA was detected in a small proportion of the loops of Henle of the renal medulla (Fig. 1G). In contrast, the rats of both the stone group and takusha group demonstrated a marked increase in OPN mRNA expression, however, the increase of the takusha group was relatively weak compared with the stone group (Fig. 1H, I).

The examination of 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 the control group rats (Fig. 3). In the stone group, the rat kidney OPN mRNA signal was clearly enhanced. The enhancement started at least 7 days after the start of drug administration. The OPN mRNA expression in the takusha group was enhanced, but was relatively weak compared with the stone group.

Discussion

As far as stone prophylaxis is concerned, patients with urolithiasis tend to be managed with the same medicine

Table 1 Summary of serum values and 24-hour urinary excretions. Values are means ± SD. *Cr* creatinine, *BUN* blood urea nitrogen, *Ca* calcium, *P* phosphorus

		Serum biochemical data (mg/dl)				24-Hour urinary excretion (mg/day)	
		Cr	BUN	Ca	P	Ca	Oxalate
Control group	Day 0 ($n = 5$)	0.2 ± 0.13	20.1 ± 1.9	10.7 ± 0.36	11.1 ± 1.55	1.08 ± 0.84	0.23 ± 0.13
	Day 7 ($n = 5$)	0.2 ± 0.11	18.8 ± 3.1	10.6 ± 0.47	11.4 ± 1.13	1.22 ± 1.02	0.26 ± 0.11
	Day 14 ($n = 5$)	0.2 ± 0.09	20.2 ± 3.2	11.1 ± 0.64	11.9 ± 1.84	1.62 ± 0.63	0.25 ± 0.15
	Day 28 ($n = 5$)	0.2 ± 0.04	20.1 ± 2.1	10.8 ± 0.49	11.4 ± 1.35	1.12 ± 0.44	0.21 ± 0.21
Stone group	Day 0 ($n = 5$)	0.2 ± 0.11	21.3 ± 2.3	10.6 ± 0.47	11.4 ± 1.30	1.12 ± 0.54	0.23 ± 0.16
	Day 7 ($n = 5$)	0.6 ± 0.32**	28.3 ± 6.2	11.8 ± 1.15	11.7 ± 2.31	4.23 ± 2.80*	0.88 ± 0.41*
	Day 14 ($n = 5$)	0.7 ± 0.23**	32.3 ± 6.3	12.0 ± 1.30	11.5 ± 1.85	5.28 ± 2.21*	1.02 ± 0.47*
	Day 28 ($n = 5$)	0.8 ± 0.44**	31.3 ± 5.3	14.0 ± 1.32*	11.8 ± 2.33	5.63 ± 1.82**	0.99 ± 0.54*
Takusha group	Day 0 ($n = 5$)	0.3 ± 0.08	20.0 ± 1.9	10.7 ± 0.47	11.8 ± 1.44	1.02 ± 0.44	0.22 ± 0.17*
	Day 7 ($n = 5$)	0.7 ± 0.28**	26.5 ± 4.8	11.0 ± 0.85	11.3 ± 1.15	3.46 ± 2.49*	0.82 ± 0.51*
	Day 14 ($n = 5$)	0.8 ± 0.38**	29.1 ± 5.1	11.8 ± 1.30	11.1 ± 1.56	4.46 ± 2.30*	0.94 ± 0.42*
	Day 28 ($n = 5$)	0.7 ± 0.41**	28.5 ± 4.9	12.8 ± 0.92*	10.8 ± 1.67	6.46 ± 2.51*	0.83 ± 0.27*

* $P < 0.05$, ** $P < 0.01$: significantly different compared with the control group on the same day after the drug administration

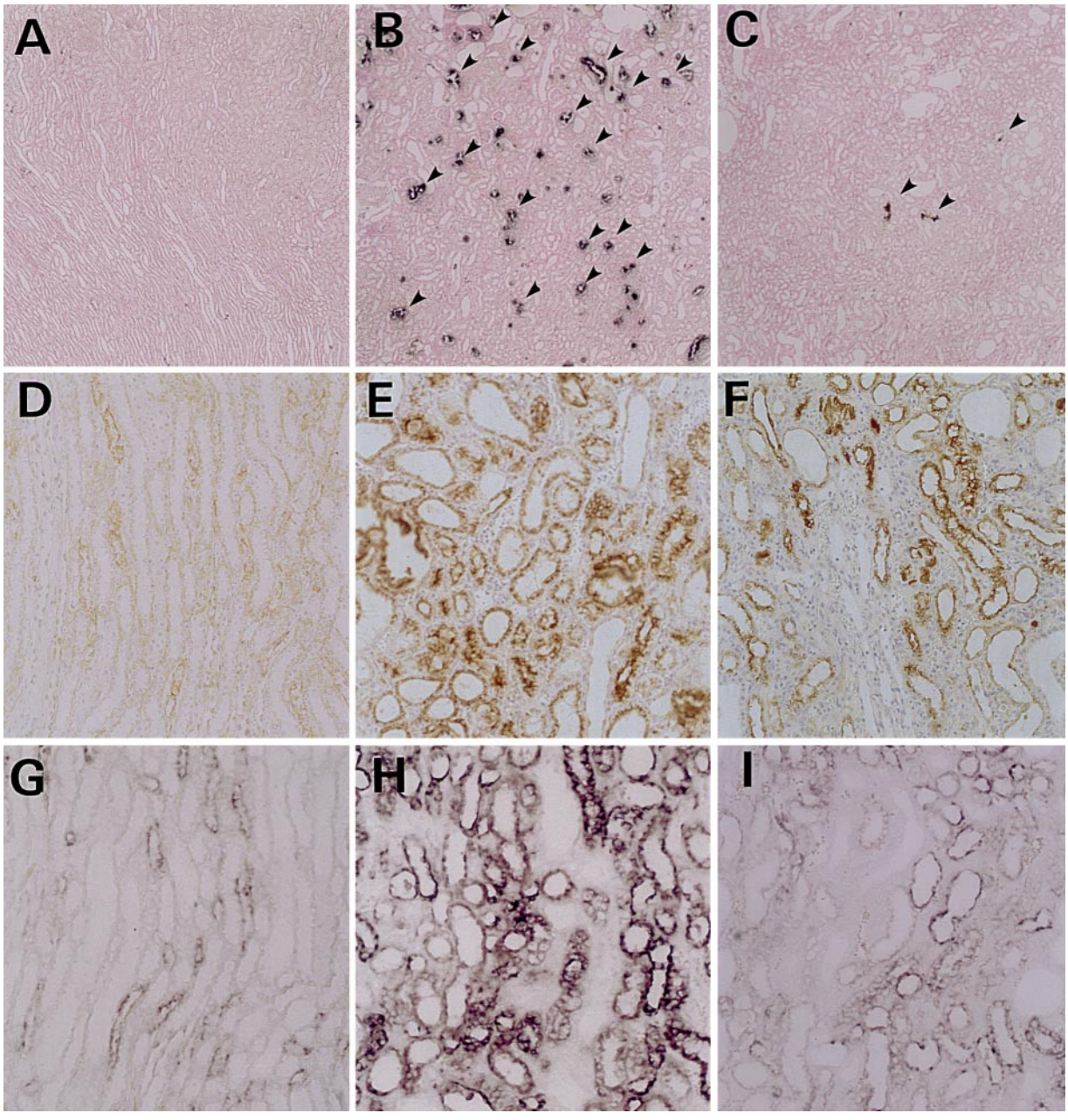

Fig. 1 Microscopic appearance of rat kidneys in the control group (**A, D, G**), stone group (**B, E, H**) and takusha group (**C, F, I**) at day 28 of treatment. Pizzolato's method [18] detected calcium oxalate deposition (*arrowheads*) (**A, B, C** × 40). Immunohistochemical staining using OPN monoclonal antibody (brown) (**D, E, F** × 200). In situ hybridization analysis of OPN mRNA (**G, H, I** × 200)

for long periods, and the medicine's side-effects as well as its efficacy may therefore become a problem. Kampo medicines have fewer side-effects than Western medicine. Unfortunately, however, the pharmacological effects of kampo medicine have not been objectively evaluated using modern scientific methods. It was recently reported that takusha exhibited inhibitory activity on calcium oxalate crystallization in an in vitro model [11]. In the present study, we evaluated the effectiveness of takusha on stone formation and OPN expression in an in vivo model.

We administered low-dose EG and vitamin D_3 to rats through a stomach tube to induce calcium oxalate stones in the kidney. We chose this experimental model because the renal failure thus produced is not severe, and the stones build up slowly and gradually. Gavage feeding ensures more accurate dosage than adding the medicine to the animals' drinking water. We administered about five times more takusha than would be a general clinical dose per weight for humans into the rats considering their body surface. The composition of calculi induced by this method was considered to be calcium oxalate, partially containing calcium phosphate, from observa-

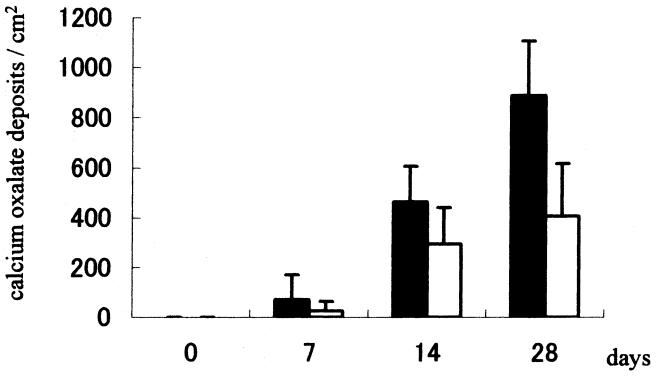

Fig. 2 The number of calcium oxalate deposits in rat kidneys examined with Pizzolato's method in the stone group (*black columns*) and takusha group (*open columns*). The data show the number of calcium oxalate deposits detected/cm^2 (cut area)

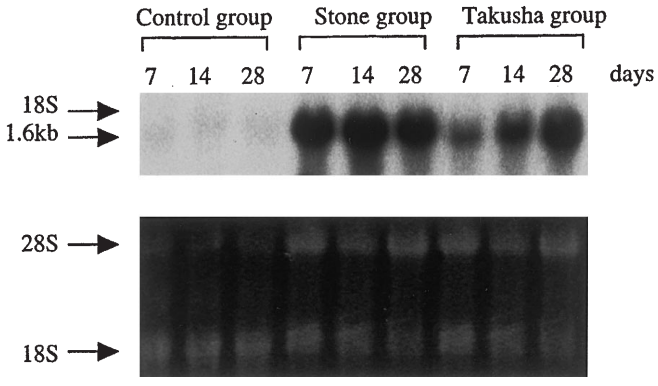

Fig. 3 Northern blot analysis of the level of OPN mRNA of rat kidney in the control group, stone group and takusha group. The transcription of an approximately 1.6 kb mRNA was detected. Equal amounts of RNA were analyzed as is evident from the ethidium bromide-stained nitrocellulose blot. The mRNA probed for OPN (*top*) in relation to the 28S and 18S ribosomal RNA bands (*bottom*) are shown. The arrows indicate 1.6 kb and 18S

tion of staining with Pizzolato's method and von Kossa's method. The results showed that the takusha administration prevented calculi formation and OPN expression.

Certain noncollagenous and plasma proteins, including OPN, sialoproteins, albumin and α_2HS-glycoprotein [2, 6] known to accumulate in bone and other mineralized tissues are also found in human kidney stones. Of these, OPN is the most prominent constituent of the calcium oxalate-associated crystal "ghosts" observed in the nuclei, lamellae, and striations of organic matrix material in deposits within epithelial cells [14]. 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 [14]. Shiraga et al. [20] 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. 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 what role OPN plays in renal stone formation in vivo [9, 10]. 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 attains the characteristic of enhancing crystallization, possibly by the apposition of ions [19].

A recent study suggested that increased nucleation of calcium oxalate monohydrate (COM) crystals in the nephron lumen and the ensuing crystal–cell interactions could upregulate OPN gene expression and protein secretion by renal tubular cells [13]. Enhanced OPN production by renal cells after an interaction with a COM crystal could have other important biological consequences, especially if OPN is released across the basolateral plasma membrane into the interstitium [5]. In any case, OPN expression is enhanced in the process of stone formation, and observations of OPN expression are useful to confirm the process of stone formation and the efficiency of a drug for the inhibition of stone formation.

The mechanism underlying the inhibition of stone formation by takusha is unclear, but it may be that, influenced by takusha, substances that inhibit stone formation are increased in the urine, and/or substances that enhance stone formation are decreased.

In this study, the OPN expression in the stone group had already increased at 7 days after the start of drug administration, when calculi were just forming. This suggested that OPN expression was promoted by the EG and vitamin D_3 administration. The OPN expression in the takusha group was weak compared with the stone group, and rose little by little, behind the stone group. This result suggests that the inhibition of OPN expression is a mechanism underlying the inhibition of stone formation by takusha.

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

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