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 vivo and in vitro, 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 matrix protein. Control rats were treated with saline while rats of the stone group were treated with ethylene glycol (EG) and vitamin D₃, 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 D₃. 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

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 hypercalcemia 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 D₃ 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 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 D3 (1α(OH)D3, alfalcaldol) (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 (Uralty, Nippon Chemipharm, Tokyo), a compound of sodium citrate and potassium citrate (Na: 4.5 mM/g, K: 4.5 mM/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 D3 were not administered). 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 D3 (stone group), (3) rats administered EG, vitamin D3, and citrate (0.5 g/kg per day citrate) (low-dose group), and (4) rats administered EG, Vitamin D3, 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 NH4OH solution. To this, we added 0.4 ml of 2 mg/ml Na2C2O4 solution and 0.4 ml of 100 mg/ml CaCl2 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 NH4OH or H2SO4. The mixture was then centrifuged and the precipitate was washed three times with a 6-ml saturated solution of CaC2O4 in water, and dissolved in 1 ml of 1N H2SO4 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% H2O2 and 5% AgNO3. After this illumination, the sections were lightly counterstained with hematoxylin (Hartman-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 EcoRI and transcribed with T3 RNA polymerase to generate an antisense cRNA probe. The plasmid was then linearized with Xhol 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 32P-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. MIII1B01(1) (Developmental Studies Hybridoma Bank, Iowa City, Iowa), which recognizes osteopontin, was used as the primary antibody. Deparaffinized sections were incubated in 0.3% H2O2 in methanol for 30 min, followed by washing in 0.1 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 MIII1B01(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.