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Expression of bone matrix proteins in urolithiasis model rats

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Abstract Urinary calcium stones are a pathological substance, and they show similarities to physiological mineralization and other pathological mineralizations. The expression of messenger (m) RNAs of osteopontin (OPN), matrix Gla protein (MGP), osteonectin (ON) and osteocalcin (OC) in bones and teeth has been described. We previously identified OPN as an important stone matrix protein. In addition, the spontaneous calcification of arteries and cartilage in mice lacking MGP was recently reported, a finding which indicates that MGP has a function as an inhibitor of mineralization. Here, we examined the mRNA expressions of OPN, MGP, ON, and OC in the kidneys of stone-forming model rats administered an oxalate precursor, ethylene glycol (EG) for up to 28 days. The Northern blotting showed that the mRNA expressions of OPN and MGP were markedly increased with the administration of EG, but their expression patterns differed. The OPN mRNA expression reached the maximal level at day 7 after the initiation of the EG treatment and showed no significant difference after 14 and 28 days, whereas the MGP mRNA expression rose gradually to day 28. The in situ hybridization demonstrated that the cell type expressing OPN mRNA was different from that expressing MGP. We suggest that OPN acts on calcification and MGP acts on suppression.

Key words Osteopontin · Matrix Gla protein · In situ hybridization · Urolithiasis · Calcium oxalate

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Introduction

Urinary stones are a pathological substance occasionally generated in renal tubules. Biochemical analyses of urinary stones have revealed that 1% to 5% of their weight consists of proteinous ingredients, and several reports have suggested the importance of proteins in stone formation [1]. We previously extracted the proteinous fraction from calcium oxalate and calcium phosphorus stones with ethylenediaminetetraacetic acid (EDTA) and identified one of the major components of urinary stones as osteopontin (OPN) [21]. We found a strong expression of OPN mRNA by distal tubular cells in the kidneys of stone-forming rats [20]. The biological significance of non-collagenous extracellular matrix proteins such as OPN, osteonectin (ON), matrix Gla protein (MGP) and osteocalcin (OC) has been extensively investigated not only in physiological calcifying tissue [2, 4, 5, 12, 13, 17, 29, 38, 41] but also in pathological calcifications such as atherosclerosis, breast cancer, calcifying epithelial odontogenic tumor and pleomorphic adenoma [11, 15, 16, 23, 26].

OPN is a highly acidic, 44-kDa phosphorylated glycoprotein with the amino acid sequence of Arg-Gly-Asp (RGD) that elicits the binding of integrin [29]. It is associated with the transformation process, and is markedly upregulated upon the transformation of cells [4, 5]. OPN shows high-affinity binding to hydroxyapatite [29], and appears to play a role in modulating the mineralization of calcifying tissues [27, 29]. These and other reports suggest that OPN also plays a role in cell attachment and pathological calcium deposition such as in urinary tract stones [8, 20, 21, 27, 29]. OC is a calcium-binding, low molecular weight (5.7-kDa), vitamin K-dependent matrix protein, also called BGP (bone γ-calboxyglutamic acid-containing protein) that can bind to hydroxyapatite [2, 13]. Based on studies of warfarin-treated animals, OC is speculated to regulate the recruitment and functioning of osteoclast precursors [12]. ON is a 38-kDa phosphorylated glycoprotein that

shows a high affinity to type I collagen [17, 38, 41]. Although ON also shows a high affinity to calcium and hydroxyapatite, ON is thought to be associated with tissue remodeling rather than calcification [7]. MGP is a low molecular weight (14-kDa), γ-calboxyglutamic acid-containing, vitamin K-dependent protein [14, 33]. The spontaneous calcification of arteries and cartilage in mice lacking MGP was recently reported [24], which indicates that MGP has a function as an inhibitor of mineralization.

Although the expressions of these proteins have been detected in the kidney [10, 20, 22], their biological functions are still unclear. The larger aim of our research is to determine the biological functions of these bone matrix proteins in physiological and pathological conditions in the human kidney. Toward this end, we used Northern blotting and in situ hybridization to investigate the changes in the expression levels of the mRNAs encoding these proteins during urinary stone formation (urolithiasis) in rats.

Materials and methods

Experimental animals and histological examination

All experimental procedures were performed in accordance with protocols approved by the Animal Care Committee of the Faculty of Medicine, Nagoya City University. The rat stone-forming model reported by Okada et al. [28] was carried out with minor modifications. 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, Oriental Yeast, Tokyo, Japan) was used. Thirty rats were used for the examination. To induce calcium oxalate deposits, 15 rats were administered 0.5 μg vitamin D_3 ($1\alpha(OH)D_3$, alfacalcidol, Chugai Pharmaceutical, Tokyo) resolved in 0.5 ml of middle-chain triacylglycerol (MCT) solution every other day and 0.12 ml of 5% ethylene glycol (EG) (Wako, Tokyo) in 1.0 ml of distilled water daily in two divided doses through a stomach tube (stone group). The other 15 rats were administered the same mount of MCT solution and distilled water without vitamin D_3 or EG (control rats).

All of the rats were weighed at weekly intervals, and pooled 24-hour urine samples were collected in metabolic cages 2 days before they were killed. The urine samples were collected with a cup containing hydrochloric acid to measure oxalate. The animals were killed and their kidneys were collected soon after the blood samples had been collected from the inferior vena cava under ether anesthesia at 7, 14 and 28 days after the initiation of the stone or control treatment. Serum blood urea nitrogen (BUN) and urinary calcium levels were determined with an automatic analyzer (Model 705, Hitachi, Tokyo) after centrifugation. Oxalate was measured by the method of Fraser and Campbell [9].

Resected kidneys were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.0) at 4°C overnight, dehydrated with a series of ethanol extractions and embedded in paraffin under RNase-free conditions. Serial 4-μm-thick sections were prepared and used for in situ hybridization. Microliths were stained by Pizzolato's method [32] to demonstrate calcium oxalate or by von Kossa's method [31] to demonstrate calcium phosphate. The results of these two staining methods were consistent.

The number of calcium oxalate deposits

Sagittal sections of each kidney were stained by Pizzolato's method [32]. 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 calcium oxalate deposits/cm^2 (cut area).

Probe preparation

The following complementary DNA (cDNA) clones were used as hybridization probes: mouse OPN cDNA containing a 1.2 kb fragment [27], mouse ON containing a 1.0 kb fragment [27], rat OC cDNA containing a 0.47 kb (1-470) fragment [2], and rat MGP cDNA containing a 0.48 kb (1-476) fragment [33]. OC and MGP cDNAs were obtained by the reverse transcription of mRNA from newborn rat bone tissue, followed by a polymerase chain reaction (PCR) and subcloning into pBluescript KS-plasmid. The sequencing of the resulting cDNAs was performed by the method of Sanger et al. [36]. The base sequences were identical to those of the rat OC and rat MGP cDNAs described previously. The specificity of these probes was confirmed by Northern blotting.

RNA extraction and Northern blotting

Kidney tissue samples for Northern hybridization were frozen with liquid nitrogen, and total RNAs were extracted from kidney tissues by the method of Chirgwin et al. [3]. 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 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 the staining of 28S or 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

The details of the in situ hybridization technique used here have been described previously [27]. Digoxigenin (DIG)-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 manufacturer's instructions. The hybridization of ON, OPN, OC and MGP 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; (3) the use of anti-sense RNA or the removal of anti-digoxigenin antibody. No positive signals were observed under any of these conditions.

Results

The results of the in vivo animal experiment

Bloods and urine samples were collected at 7, 14, and 28 days after the initiation of the stone or control treatment from the control rats ($n = 5$) and stone rats ($n = 5$). Biochemical analyses of the serum and urinary excretion were carried out. At 28 days, the concentration of oxalate in the 24-hour urinary excretion in the stone group

was more than four times that of the control rats (0.99 ± 0.54 and 0.23 ± 0.16, respectively). The administration of EG was considered to be effective in stone rats with this value. At the same time point (28 days), the concentrations of serum BUN showed no significant difference between the stone group and control rats (24.3 ± 5.32 and 20.1 ± 2.12 respectively), indicating that the renal failure was not severe. No renal stone was observed in the urinary tract of any animal at death.

Calcium oxalate deposits were stained by Pizzolato's method (Fig. 1A), and the number of calcium oxalate deposits per cm^2 was calculated (Fig. 1B). No stone formation was detected at 7, 14, or 28 days in the control rats. At 7 days in the stone group, calcium deposits were detected in one of the five animals. At 14 and 28 days in the stone group, calcium deposits were detected in all five animals. A linear increase in the number was observed over days 7, 14 and 28 in the stone group.

Change in the level of mRNA and the cell type during treatment

About 20 μg of total RNA was extracted from the rat kidneys at 7, 14 and 28 days, then blotted and hybridized with the ^{32}P-labeled cDNA probe (Fig. 2). ^{32}P-labeled cDNA probes encoding OPN, MGP, ON and OC were prepared to determine the cell types expressing the mRNAs of these bone matrix proteins. Paraffin sections were hybridized with DIG-UTP labeled cRNAs encoding their cDNAs (Fig. 3).

The expression of OPN was detected by Northern blotting in the control rats as a faint signal of 1.6 kb (Fig. 2). At day 7, the intensity of this signal was markedly increased to a maximal level by the adminis-

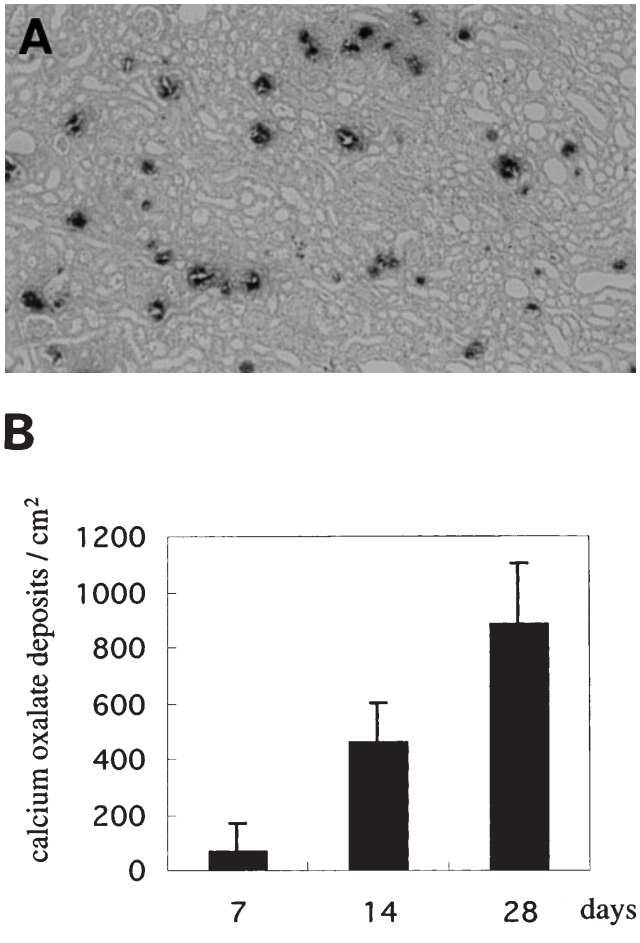

Fig. 1 **A** Pizzolato's method detected calcium oxalate deposition in the kidneys of the stone group at day 28 of treatment (original magnification ×40). **B** The number of calcium oxalate deposits in rat kidneys examined with Pizzolato's method in the stone group. No calcium deposits were detected in the control rats. The data (mean ± SD, $n = 5$) show the number of calculi detected/cm^2 (*cut area*)

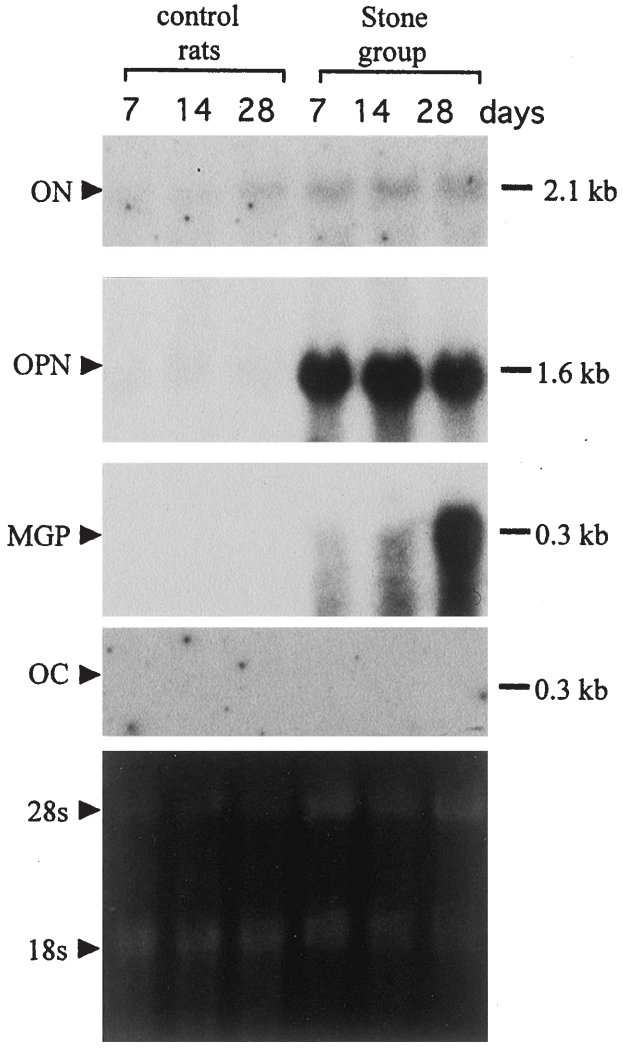

Fig. 2 Northern blot analysis of the level of mRNAs of kidneys in the control rats and stone group. Expressions of osteonectin (ON), osteopontin (OPN), and matrix gla protein (MGP) were observed. Equal amounts of RNA were analyzed, as is evident from the ethidium bromide-stained nitrocellulose blot. The mRNA probed for ON, OPN, MGP and osteocalcin (OC) (top) in relation to the 28S and 18S ribosomal RNA bands (bottom) are shown

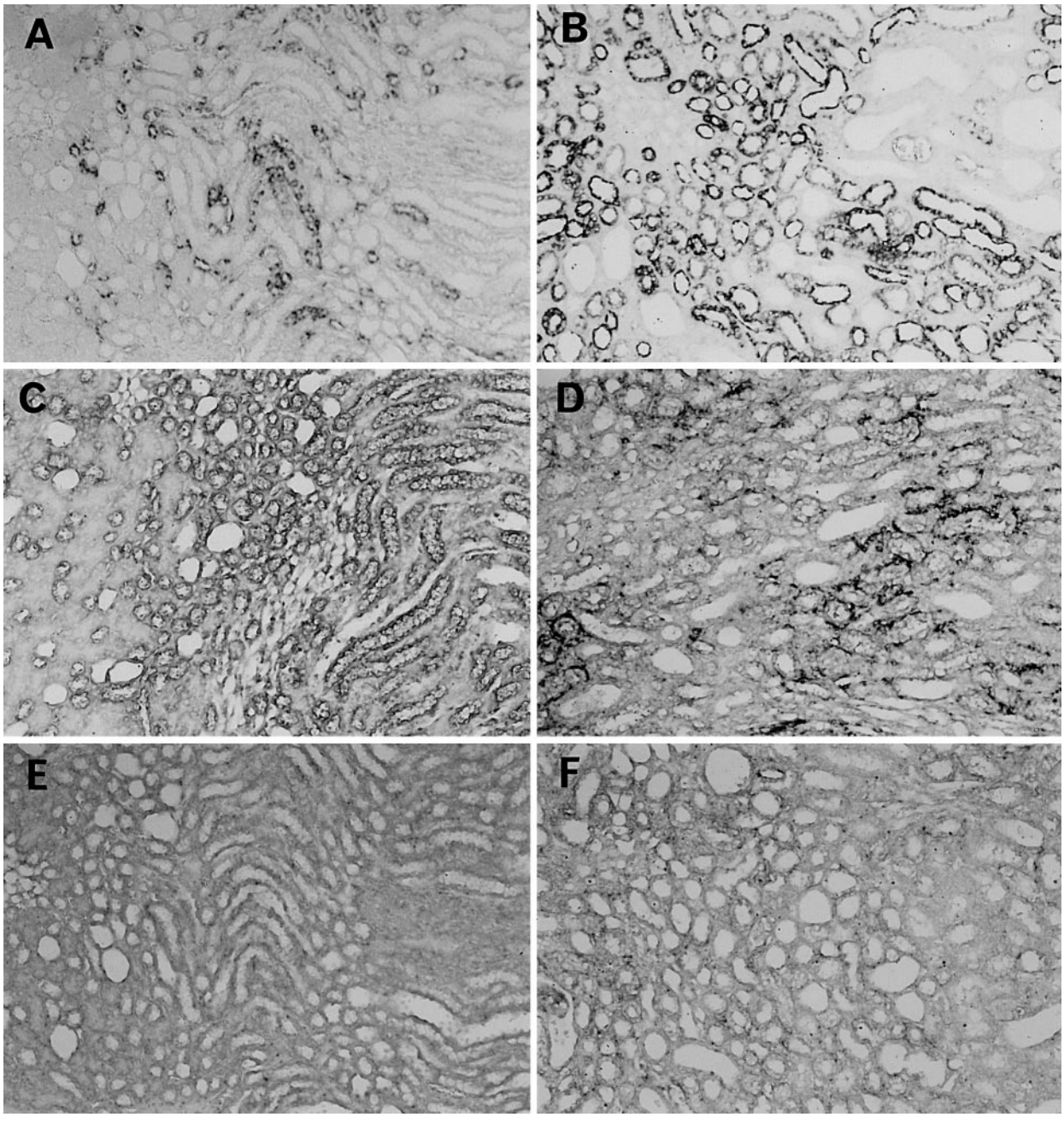

Fig. 3A–F Microscopic appearance of the outer medulla of kidneys of the control rats (**A**, **C**, **E**) and stone group (**B**, **D**, **F**) on day 28 of treatment. In situ hybridization analysis of OPN mRNA (**A**, **B**), MGP mRNA (**C**, **D**), ON mRNA (**E**, **F**). (Original magnification ×100)

tration of EG, and showed no further significant difference at 14 and 28 days. The cell type expressing OPN mRNA was demonstrated by in situ hybridization (Fig. 3A, B). In the kidneys of the stone group rats, the epithelial cells in both the distal and proximal tubules, the loops of Henle and the collecting ducts, and particularly the distal tubular cells of the renal medulla expressed OPN mRNA (Fig. 3B). In contrast, in the kidneys of the control rats, only a small population of the epithelial cells in the distal tubules showed OPN mRNA-positive signals (Fig. 3A).

The expression of MGP was detected as a faint signal in the control rats by Northern blotting (Fig. 2). The intensity of the signal was increased by the administration of EG at day 7, and rose gradually to day 28. The cell type expressing MGP mRNA was demonstrated with in situ hybridization (Fig. 3C, D). In the kidneys of the control rats, MGP mRNA was detected in the epithelial cells of proximal cortical tubules and medullary tubules (Fig. 3C). In contrast, in the stone group, a large population of the epithelial cells in the cortical tubules showed MGP mRNA-positive signals (Fig. 3D). The epithelial cells in which MGP mRNA was detected were

not similar to the epithelial cells in which OPN mRNA was detected.

The expression of ON was detected as a faint signal in the control rats by Northern blotting (Fig. 2). The intensity of the signal was increased at day 7 by the administration of EG and showed no significant difference at days 14 and 28, but the increase was mild compared with those of OPN and MGP. ON mRNA was detected by in situ hybridization in the epithelial cells of distal cortical tubules and the epithelial cells lining nearly all medullary tubules, both the collecting tubules and Henle's loops (Fig. 3E, F). The expression of OC was not detected in the kidneys of the control rats or the stone group by Northern blotting (Fig. 2) or by in situ hybridization (data not shown).

Discussion

In the present study, we used Northern blotting and in situ hybridization to examine the expression of the genes encoding the bone matrix proteins OPN, MGP, ON, and OC in the process of stone formation in rats administered EG (an oxalate precursor) and vitamin D_3. This experimental model with low-dose EG and vitamin D_3 administered through a stomach tube to induce calcium oxalate deposits in the rat kidneys was chosen because (1) the stones build up slowly and gradually, and (2) the stomach tube administration route is more effective and more exact than mixing the EG/vitamin D_3 medicine into the rats' drinking water. The component of calculi induced by this method was calcium oxalate, with some calcium phosphate, based on the findings obtained with staining by the Pizzolato and von Kossa methods.

Fraser and Price [10] showed MGP mRNA expression in the rat kidney by Northern blotting and immunohistochemically detected MGP protein in the epithelium of Bowman's capsule and the proximal tubules. In the present study, in situ hybridization with a DIG system detected MGP mRNA within epithelial cells of the proximal cortical tubules and medullary tubules. Kopp et al. [22] showed ON mRNA by Northern blotting; ON protein was detected within epithelial cells of distal cortical tubules (but not within the proximal tubules or glomeruli) and within epithelial cells lining nearly all medullary tubules, both collecting tubules and Henle's loops by immunohistochemical staining. We detected ON on the mRNA level in similar cells by in situ hybridization. We did not detect OC mRNA by in situ hybridization or by Northern blotting.

Here, OPN mRNA was detected in the control rats in some of the distal tubular cells in the outer medulla by in situ hybridization. OPN mRNA was detected in the rats administered EG in the greater part of the tubular cells in the outer medulla and in tubular cells in the cortex. The tubular cells in which OPN mRNA was detected were radial in the cortex. MGP mRNA was detected in the kidneys of rats administered EG, in the outer medulla, but the cells in which MGP mRNA was detected were not similar to the cells in which OPN was detected.

The actions of OPN that can be demonstrated in vitro are myriad, consistent with a diversity of physiological roles for this protein. OPN is chemotactic for smooth muscle cells and macrophages. In bone, phosphorylated OPN is thought to be the natural ligand for the osteoclast integrin $\alpha_v\beta_3$. This integrin is abundant in osteoclasts and plays a role in osteoclast activation [6, 34]. Integrins are not only involved in cell attachment but are also signal-transducing receptors. A signaling role for OPN is suggested by its action in modulating intracellular calcium in $\alpha_v\beta_3$-containing cells [25], and by its action in inducing chemotaxis in cells transfected with CD44 [39]. More recently, it was shown that the amino-terminal OPN fragment contains a cryptic binding site that can be recognized by $\alpha_9\beta_1$ integrin [37]. OPN is also a regulator of gene expression. For example, in mouse kidney epithelial cells, osteopontin inhibits the induction of nitric oxide synthase gene expression by an inflammatory mediator [18]. It has been suggested that the diverse features and functions of OPN reflect, as a common element, roles of this protein in inflammation and tissue repair [34]. The renal expression of OPN is enhanced in the recovery from ischemia [30] and in renal development [35].

In the present study, Northern blotting showed that the expression patterns of the MGP, OPN, and ON mRNAs differed. The ON mRNA expression was slightly increased at day 7 by the administration of EG and showed no significant difference at day 14 and 28 from the level at the initiation of the EG administration. The OPN and MGP mRNA expressions were greatly increased by the administration of EG. We observed a time-dependent increase of MGP mRNA in the rat kidney, but not of OPN mRNA. The OPN mRNA expression had already reached a peak at day 7 following the EG administration. At day 7, stones are just forming, as detected by microscopy.

The Northern blotting suggested that the possible reasons for the time lag of the MGP expression behind that of OPN were that MGP expression is enhanced (1) by only the toxicity of oxalate, (2) due to the expression of some protein related to stone formation, or (3) due to the direct attachment of calcium deposits. We suspect that the reason is that MGP expression is enhanced due to the expression of OPN or another protein related to stone formation. If the MGP expression is enhanced by the toxicity of oxalate, the MGP expression should reach the maximum at day 7 from the initiation of the EG administration, but in fact did not in this study. If the MGP expression is enhanced due to the direct attachment of calcium deposits, the site of MGP expression must be the same as that of the deposition, but was not the same in our observations. We suggest that the accumulation of proteins is responsible for the time lag between the initiation of EG and the expression of MGP.

Though both OPN and MGP are bone matrix proteins and are detected in bone formation in embryonic

and adult mammalian bones, the cells expressing these two proteins are not the same. In bone formation, OPN mRNA has been detected in osteoblasts, osteocytes, and hypertrophic chondrocytes [27, 40]. MGP mRNA has been detected in chondrocytes and hypertrophic chondrocytes [14, 19]. Osteoblasts, which express only OPN and not MGP, act on calcification or bone formation. Chondrocytes, which express only MGP and not OPN, never act on calcification or bone formation. Hypertrophic chondrocytes, which express both OPN and MGP, cannot form calcifications, but when they mature they can acquire calcification ability. The calcium-binding phosphoproteins such as OPN are thought to have an important role in mineralization, and our previous study suggested that OPN protein may also have a significant role in calcium stone formation, not only of calcium phosphate but also calcium oxalate stones. The spontaneous calcification of arteries and cartilage in mice lacking MGP [24] indicates that MGP has a function as an inhibitor of mineralization. We suspect that another function of MGP is as an inhibitor of stone formation. We suggest that the balance between OPN and MGP in tubular cells controls stone formation, similar to calcification.

In conclusion, we observed the mRNA of the bone matrix proteins OPN, MGP, ON, and OC with in situ hybridization and Northern blotting in the kidneys of rats administered EG, as a urolithiasis model. The expressions of OPN, MGP and ON mRNA were enhanced in the rats administered EG. We suggest that OPN acts on calcification and MGP acts on suppression.

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