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X. J. Jiang · T. Feng · L. S. Chang · X. T. Kong
G. Wang · Z. W. Zhang · Y. L. Guo

Expression of osteopontin mRNA in normal and stone-forming rat kidney

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Abstract Human urine contains several macromolecules which inhibit calcium oxalate crystallization. Osteopontin (or uropontin), a secreted phosphoglycoprotein with the amino acid sequence Arg-Gly-Asp (RGD) and high affinity to hydroxyapatite, is one such inhibitor. To investigate the action of this protein on renal stone formation, the expression osteopontin gene in normal and chemically induced urolithiasis rat kidney was compared at both mRNA and protein levels. Northern blot analysis shown a significant increase of osteopontin mRNA level in stone-forming rat kidney compared with normal ones. In an in situ hybridization study, we localized the transcripts of the osteopontin gene in epithelial cells of both distal and collective tubules, and found a remarkably strong signal in stone-forming rats. The amount and distribution of the protein in kidney from immunocytochemistry staining showed the same pattern as seen in situ hybridization. These findings indicate that osteopontin may be an important macromolecule in the normal endogenous defence against the formation of urinary calculi.

Key words Nephrolithiasis · Calcium oxalate · Osteopontin · Macromolecule inhibitor · Rat kidney

Introduction

Urolithiasis is a common human malady, with the majority of stones formed in the urinary space mineralized with calcium oxalate. The chemical nature of the disease is poorly understood. Urine contains inhibitors of stone mineral crystal growth [4]. They may be responsible, in part, for the relative infrequency of nephrolithiasis and urolithiasis in patients and healthy subjects despite the urine being frequently supersaturated with calcium oxalate [8]. A large degree of inhibition of CaOx crystallization in normal urine stems from protein macromolecules [12, 17, 31]. These proteins may be protective by binding to the crystal's surface, preventing growth and aggregation of small crystals into calculi that could be retained in the urinary tract [13]. Recently one such macromolecule, named uropontin (UP), identical to human osteopontin (OPN), has been isolated and characterized from human urine. In vitro studies have shown that the protein is a potent inhibitor of CaOx crystallization [28, 30]. Although the precise role of uropontin has not been defined, it seems likely that abnormalities in the secretion or processing of the protein may play a role in the pathophysiology of nephrolithiasis. Because it has been cloned and sequenced [5], osteopontin has been studied somewhat more extensively than any other protein inhibitor [15, 16, 25, 32, 33]. However, investigations of the cellular and subcellular distribution of the protein within the kidney have yielded some conflicting results [15, 16, 25, 26]. In the murine kidney, OPN mRNA and protein were detected only in a subset of nephrons and was localized to the thick ascending limbs of Henle and distal convoluted tubule [22]. In rat kidney, Kleinman et al. [15] observed osteopontin and its message at two sites in the rat kidney: papillary surface epithelium (PSE) and descending thin limbs of Henle (DTL). Kohri et al. [16], in a study in which they show increased expression of the protein in a rat model of nephrolithiasis, detected OPN and its mRNA at distal tubular cells in both groups of rats. The increase in osteopontin mRNA expression by distal tubular cells and collecting duct cells was associated with the dosage and the duration of administration of stone-inducing drugs. Neither normal rats nor stone-forming rats' kidney have a detectable osteopontin mRNA signal at glomeruli and proximal tubular cells. While the majority of studies have showed that OPN as a inhibitor of CaOx crystallization, there are nevertheless suspicions that OPN might act as a promoter in the formation of

X. J. Jiang (✉) · T. Feng · L. S. Chang · X. T. Kong · G. Wang
Z. W. Zhang · Y. L. Guo
Institute of Urology, First Hospital, Beijing Medical University,
1 Da Hong Luo Chang St., Beijing 100034, P. R. China

urinary calcium stone nuclei because it has been found in major matrix components of renal calculi and upregulated expression in an experimental rat model of renal stone formation [23, 33]. All those inconclusive results motivated us to do the following study. We hypothesized that osteopontin might be upregulated in rats with nephrolithiasis due to feeding ethylene glycol. In this setting, the increased OPN expression appears as a normal defence of counterbalancing hyperoxaluria. Our objective was to examine whether expression and the localization of osteopontin between normal and stone-forming rat kidney are different.

In the study, using mouse monoclonal antibody against rat osteopontin and digoxigenin-labelled single-strand RNA probes, we investigated the immunocytochemical expression of osteopontin and its mRNA in both groups of rats. We found a marked increase in osteopontin mRNA and the protein in renal distal and collective tubular epithelial cells in a rat model of urinary stones compared with normal rats. Further examination was also carried out by Northern blot hybridization. However, no changed sites of expression have been observed during the experiments.

Materials and methods

Animal procedures and tissue processing

Male Sprague-Dawley rats ($n = 10$) weighing approximately 150–200 g (purchased from Laboratory Animal Centre of Beijing Medical University, Beijing, P. R. China), were divided into two groups and kept in metabolic cage for 3 weeks prior to killing. The experimental animal model of calcium oxalate urolithiasis was induced by 1% ethylene glycol and 1% ammonium chloride administered in drinking water. Rats were killed with an intraperitoneal injection of sodium pentobarbital, left kidneys were removed and immediately frozen in liquid nitrogen for Northern blot analysis, remaining kidneys were fixed by retrograde perfusion via the abdominal aorta with 4% paraformaldehyde (400 ml for each animal), kidney samples were left for 2 h in same fixative at 4°C, and dehydrated in graded ethanol series, then paraffin-embedded.

Detection of osteopontin mRNA in kidney by in situ hybridization

Digoxigenin-labelled single-strand RNA probes were prepared using DIG RNA labelling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions. The plasmid containing rat smooth muscle osteopontin cDNA, 2B7 (a kind gift of Dr Cecilia M. Giachelli, Department of Pathology, University of Washington, Seattle, USA) was linearized with BamH I at 5′ and Hind III at 3′, and transcription was accomplished in the presence of digoxigenin-UTP with T7 and T3 RNA polymerases, respectively, to generate single-stranded sense and anti-sense RNA probes. Hybridization was carried out as follows: 5-μm sections were dewaxed by incubation in xylene (10 min × 3), then incubated in progressively decreasing concentrations of ethanol (100%, 90%, 80%, 70%), and finally in PBS (water treated by diethyl pyrocarbonate, DEPC).

Following the treatment by proteinase K for 15 min at 37°C, the sections were fixed with freshly prepared 4% paraformaldehyde for 10 min. Then they were treated with 0.2 M HCl for the inactivation of internal alkaline phosphatase and were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min., following two 3-min rinses with 0.1 M PBS. The slides were dehydrated with an ethanol series and dried. The hybridization solution contained 50% deionized formamide, 10% dextran sulfate, 1 × Denhardts solution, 600 mM NaCl, 10 mM TRIS-Cl (pH 7.6), 200 μg/ml tRNA, 0.25% SDS, 1 mM EDTA (pH 8.0), and approximately 0.4 μg/ml digoxigenin-labelled RNA probe. Hybridization solution (50 μl) was placed on each section, which was covered with parafilm and incubated at 50°C for 16 h in a moisture chamber. After hybridization, the parafilm was dislodged by washing with 5 × SSC briefly and with 50% formamide, 2 × SSC at 50°C for 30 min. RNase A treatment (5 μg/ml) was carried out at 37°C for 30 min. The sections were washed with 2 × SSC and 0.2 × SSC for 20 min twice at 50°C. Slides were blocked with 1% blocking solution for 30 min at room temperature, then incubated with antidigoxigenin antibody(1:500) at 4°C overnight. Hybridized digoxigenin-labelled probes were detected by nucleic acid detection kit according to the manufacturer's instructions. After colour reaction with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP), the slides were rinsed with TE (10 mM TRIS-HCl, 1 mM EDTA), pH 8.0 and mounted without counterstaining. Controls included: 1) hybridization either with the sense probe or with non probe; 2) RNase A treatment (10 μg/ml) prior to hybridization for 30 min at 37°C. All solutions used for hybridization and pretreatment with treated with 0.1% DEPC (Serva) and autoclaved, and all glassware was baked at 180°C for 8 h to inactivate RNase.

Detection of osteopontin in kidney by immunohistochemistry

Sections 5-μm thick were dewaxed by incubation in xylene followed by incubations in progressively decreasing concentrations of ethanol, and finally in water. Endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 30 min. Slides were blocked with 1% bovine serum albumin in PBS for 1 h, then incubated with mouse monoclonal antibody against rat OPN (Developmental Studies Hybridoma Bank, Iowa City, USA) or PBS for 30 min at room temperature. Slides were washed with PBS three times, then incubated with biotinylated goat anti-mouse IgG (Vector, Burlingame, Calif.) 1:400 for 40 min. Slides were washed with PBS, then incubated with extravidin-peroxidase (Vector) 1:500 for 40 min, then rinsed in PBS. Colour was developed with DAB, then rinsed in PBS. Sections were counterstained with Harris haematoxylin, and mounted with Permount. As controls, kidney sections were incubated without antibody against rat OPN.

Northern blot analysis

Total RNA was isolated according to the acid guanidinium thiocyanate-phenol-chloroform method, electrophoresed in 1.2% agarose–7% formaldehyde gel in 1× MOPS buffer, pH 7.0. Following electrophoresis, gel was rinsed in DEPC-treated water, after soaking with 0.05 M NaOH for 15 min, and with 20× SSC for 45 min, RNA was transferred to nylon membrane by capillary action in 20× SSC for 18 h. DNA probes were labelled with [α^{-32}P]dCTP (concentration 10 μCi/μl) by random hexamer priming (primer-a-gene labelling system, Promega). Baked filters were prehybridized for 4 h at 42°C in a hybridization solution containing 50% formamide, 6× SSC, 4× Denhardts solution, 0.5% SDS, 200 μg/ml sonicated salmon sperm DNA. Hybridization proceeded at 42°C for 36 h in the same buffer containing ^{32}P-labelled DNA probe (specific radioactivity: $\sim 10^8$ cpm/μg). Filters were then washed in 2× SSC/0.1% SDS, 0.2× SSC/0.1% SDS twice at 42°C for 20 minutes. An autoradiogram of the blot was prepared a −70°C for 24 h using radiographic film and two intensifying screens. The gel was subsequently reprobed with β-actin to verify that an equal amount of RNA was loaded in each lane.

Results

Demonstration of osteopontin mRNA and protein in a rat model of urinary stone

In situ hybridization

In the control kidney, osteopontin mRNA was detected in a small proportion of distal and collective tubular cells in the renal medulla by in situ hybridization (Fig. 1). Glomerular and proximal tubular cells were negative. In contrast, rats with renal stones induced by ethylene glycol and ammonium chloride had a marked increase in osteopontin expression in the distal and connecting duct cells of the medulla (Fig. 2). The controls showed a non-positive signal (result not given).

Immunohistochemical identification

By indirect immunoperoxidase staining, kidney tissue sections obtained from stone-forming and control rats were stained for the protein using the monoclonal antibody. In control rats, epithelial cells of the distal and connecting duct were weakly positive (Fig. 3). Rats with renal stones showed a significant increase in intensity of immunoreactive osteopontin in the same sites (Fig. 4). The controls have a non-positive staining (result not listed).

Northern blot analysis

Using a labelled rat smooth muscle OPN cDNA probe for Northern blot analysis. A faint signal was observed in the control kidney (Fig. 5). The level of OPN mRNA in stone-forming rats was 9.3-fold higher (mean comparison) than that in control rats (Fig. 5, Table 1).

Polarization microscopy

This illustrated renal stone formed in rats kidney induced by ethylene glycol and ammonium chloride (Fig. 6). Crystals were mainly restricted to renal medulla tubular lumen.

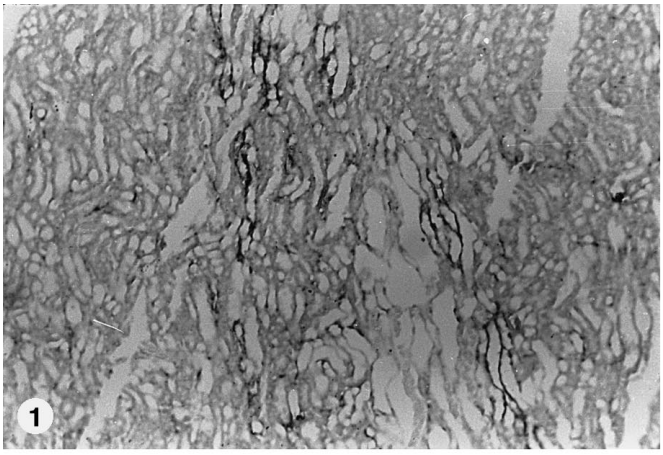
Discussion

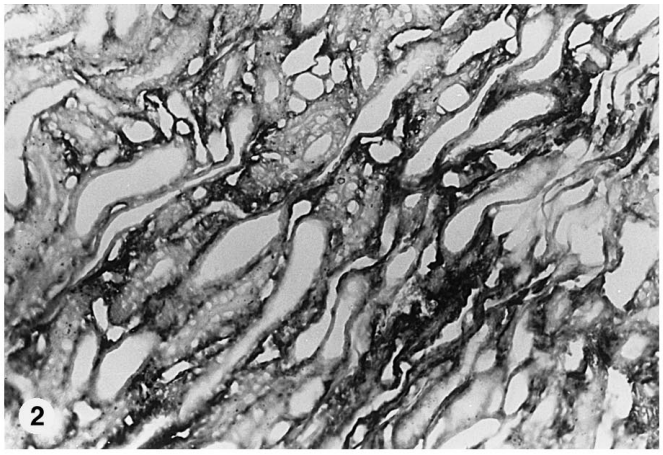
The majority of urinary stones are made up of calcium oxalate crystals and the process of crystallization can be influenced by various factors including urinary proteins. In recent years, several urinary protein have been isolated and characterized including Tamm-Horsfall protein (THP) [12], nephrocalcin (NC) [24], inter-α-trypsin inhibitor [29], uronicacid-rich protein (UAP) [1], and uropontin (UP) [28].

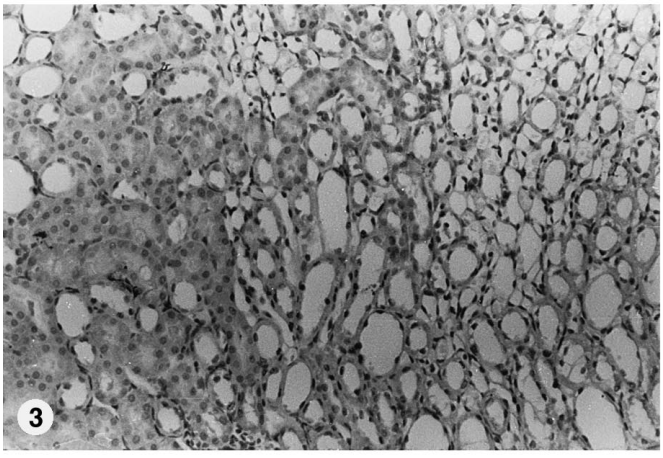
UP was initially purified from human urine because of its capacity to inhibit calcium oxalate crystallization [28]. Since then, expression of UP has been identified in mouse [22] and rat kidney [15, 16, 26, 33] by several investigators. But there is controversy regarding which nephron segments demonstrate OPN and its mRNA.

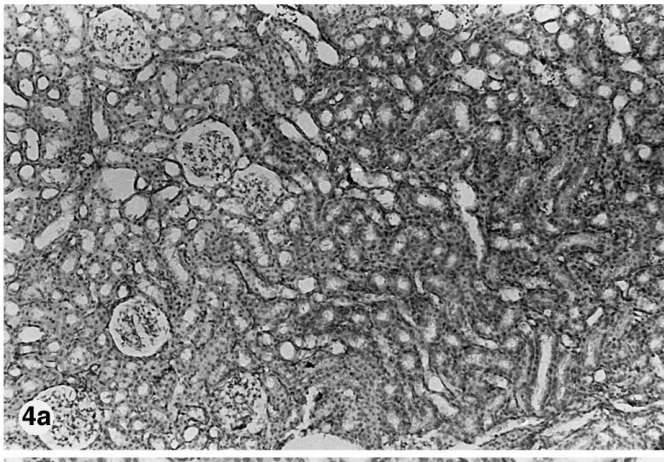
Our current study clearly shows OPN protein by immunohistochemistry and its message by in situ hybridization at epithelial cells of distal and collective ducts in rat kidney, which is nearly the same as Kohri's, but different from Kleinman's. Ullrich et al. [32], using an antibody raised against an OPN-related 20-kDa protein secreted by the MDCK cell line, performed immunocytochemistry on rat kidneys. They found staining in all parts of the nephron, including DTL at its origin from PCT S3 and inner medullary-collecting duct. In an experimental rat model of renal stone formation without renal failure, Umekawa et al. [33] found OPN mRNA sporadically in both the distal and proximal convoluted tubule and loops of Henle in both groups of rat kidney with a stronger expression in stone-forming rats. Giachelli et al. [9] showed an increased expression of the protein in a model of tubulointerstitial nephritis. They demonstrated, although occasional weak staining was observed in some tubular epithelial cells of the distal tubules and parietal epithelial cells of Bowman's capsules and cortices, that most cortical structures were negative. In the medulla, a much higher level of staining was found in the tubular epithelium, collecting ducts and uroepithelial lining cells. The reasons for the differences between our study and those referred to above are not completely apparent. We postulated it might lie in the following aspects: (1) OPN expression is different in different species (in a following experiment, we detected OPN at proximal, distal, Henle' s loops and collecting ducts, but found the message mainly at the sites of interstitium and collective tubule of medulla in human kidney. Results not listed). (2) Since OPN expression by epithelial cells varies with age [6], and is altered in degenerative kidney diseases such as angiotensin II-induced tubulointersitial nephritis and experimental hydronephrosis [7, 9], it might be reasonable to speculate that renal OPN expression is subject to change in different renal disorders. (3) Methods with tissue preservation and kidney fixation may account for some of the differences in localization for OPN and OPN mRNA. We very much doubt that some parts of kidney do secrete OPN, and cannot be detected it in normal conditions, but it could be stained for OPN when renal disease occurred. (4) OPN can be secreted by different kinds of cells in kidney [6], and whose expression has been shown to be affected by a variety of factors, which might also contribute to the controversy [5, 6, 13].

Studies have indicated that a calcium oxalate monohydrate (COM) crystal in the distal nephron lumen could interact with anionic sialic acid-containing structures on apical cell surface microvilli, and utilizing pri-









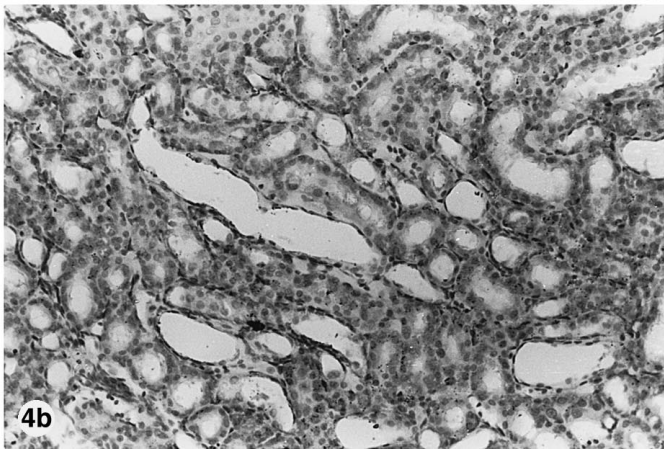


Fig. 1 In situ hybridization for mRNA in the kidney of control rats. Distal and collective tubular epithelial cells in the renal medulla were sporadically positive. (Original magnification: ×100.)

Fig. 2 In situ hybridization for mRNA in the kidney of stone-forming rats. Staining in distal and collective tubular epithelial cells was much stronger. (Original magnification: ×200.)

Fig. 3 Immunostaining for osteopontin in the kidney of control rats. Distal and collective tubular epithelial cells in the renal medulla were sporadically positive. (Original magnification: ×200.)

Fig. 4a, b Immunostaining for osteopontin in the kidney of stone-forming rats. Staining in distal and collective tubular epithelial cells was much stronger. (Original magnification: ×100.)

mary cultures of rat inner medullary collecting duct cells suggests that COM crystals bind to specific receptors on the cell surface [18, 19]. If renal tubular cells in vivo behave as renal cells do in culture, anchoring of newly formed COM crystals to apical microvilli could promote crystal retention in the kidney either through continued crystal growth/and by aggregation of additional crystals. Anionic molecules in tubular fluid, including specific glycoproteins such as OPN, could impede this cascade by coating the crystal and thereby preventing its adhesion to the cell. Lieske et al. [20] employed cultures of renal epithelial cells (BSC-1 and MDCK lines) and COM crystals as a model system to study the interaction between kidney cells and crystals in tubular fluid (both cell lines are probably derived from the distal nephron) [10, 18, 34]; they found COM crystals added avidly to the medium-bound osteopontin. Northern blotting showed that the constitutively expressed gene encoding human OPN was maximally stimulated in BSC-1 and MDCK lines after exposure to COM crystals for 12 h, while OPN mRNA by 3T3 fibroblasts was unchanged. In a renal injurious model induced by gentamicin, change in the expression of OPN mRNA was not observed, while expression of the mRNA in stone-forming

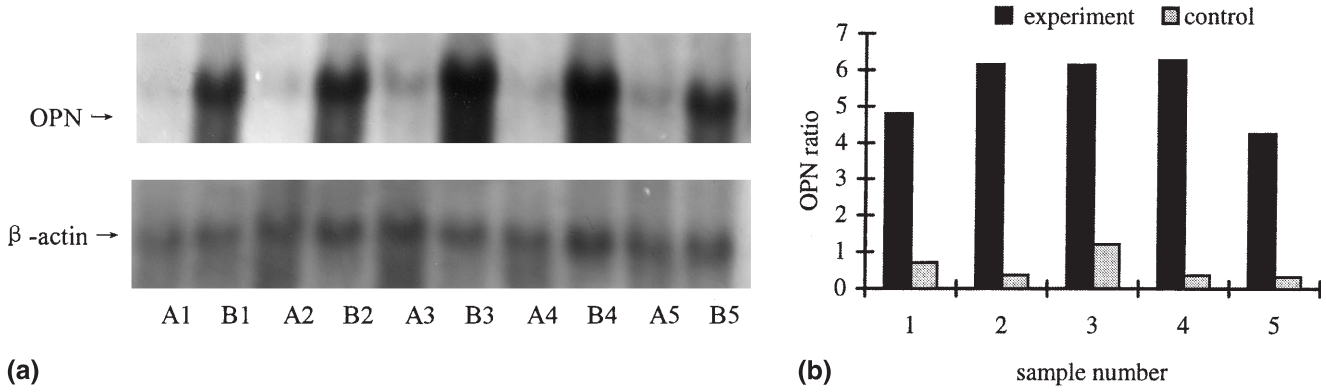


Fig. 5a Total RNA (40 μg/lane) from normal and stone-forming rat kidney was electrophoresed, blotted to nylon membrane, probed with osteopontin (OPN) and β-actin cDNA, and assessed by autoradiography (B1–B5 is stone-forming rats, A1–A5 is control rats). **b** Graphic representation of the OPN ratio relative to β-actin (loading control), obtained after densitometry of autoradiograph

Table 1 Densitometry of autoradiographs for Northern blot analysis

Sample	Experimental group			Control group		
	OPN	β-actin	Ratio	OPN	β-actin	Ratio
1	0.48	0.10	4.80	0.07	0.10	0.70
2	0.80	0.13	6.15	0.05	0.14	0.36
3	0.92	0.15	6.13	0.12	0.10	1.20
4	0.69	0.11	6.27	0.07	0.20	0.35
5	0.68	0.16	4.25	0.06	0.19	0.32

Densitometry of autoradiographic bands were analysed by Molecular Analyst Software for Bio-Rad's Imaging Analysis Systems. Ratio value (relative to loading control) was tested using the *t*-test. Difference between stone-forming and control rats was significant ($P < 0.001$)

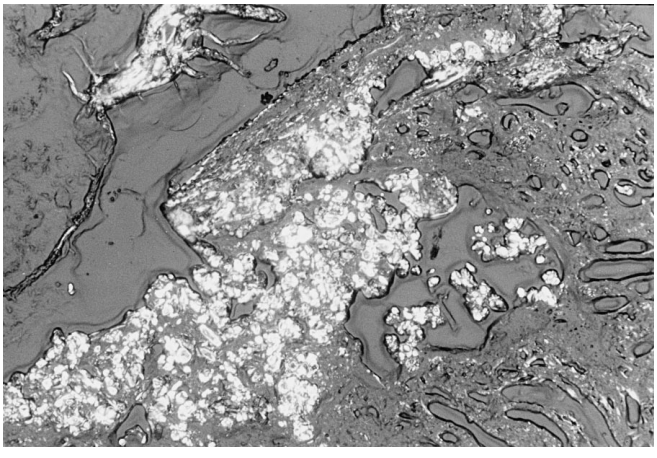


Fig. 6 Polarization microscopy illustrating renal stone formed in rat kidney induced by ethylene glycol and ammonium chloride. (Original magnification: ×40.)

rats kidney induced by glyoxylic acid was enhanced [33]. All these results might suggest COM and/or oxalate appear to be a somewhat unique stimulator for osteopontin expression of renal epithelial cells. Our Northern blot and in situ hybridization showed similar results to Kohri's [16], OPN mRNA in renal stone rats is markedly increased, 9.3-fold higher than that in non-renal stone rats. At the same time, a marked labelling for OPN of secretory granules in distal and collecting ducts was also observed. This evidence suggested increased nucleation of COM crystals in the nephron lumen and the ensuing crystal–cell interactions could upregulate OPN gene expression and protein secretion by renal tubular cells. Since OPN can block adhesion of COM crystals to renal epithelial cells [21], increased production of the glycoprotein and its secretion into the nephron lumen would decrease adhesion of additional crystals to the cell surface, thereby protecting the kidney against crystal retention and eventual nephrolithiasis. Because the urine in the distal ducts is usually the most concentrated, it should be noted that crystal-stimulated renal cell OPN secretion might represent an effort on the part of cells to counterbalance the increased presence of this particulate material in tubular lumens.

Tissue culture and renal stone-forming rat kidney studies have provided evidence that both CaOx crystals and oxalate ions are injurious to renal epithelial cells [14]. In this setting, the binding of OPN to the $\alpha_v\beta_3$ integrin, which is present on the surfaces of some cells, would immobilize the protein. The integrin is not normally accessible to OPN within the urinary space, since it is present on basolateral surfaces of tubular cells in the normal kidney. However, epithelial cell injury, including that induced by crystalluria, may disrupt cell polarity and redirect this integrin to an accessible site on luminal surfaces [27]. Attachment of CaOx crystals to OPN on the injured epithelium could support stone formation by promoting the retention of crystals within the kidney [13]. Continued accretion of OPN and possibly other proteins at the surface of growing calculi would lead to the characteristic lamellar pattern typical of crystal formation. So the protein might play dual roles in crystallization, exerting a net inhibitory effect in the fluid phase, but serving as nucleation sites after immobilization [3]. Furthermore, As there were tests showing that decreased phosphorylation reduced the protein's inhibitory capacity [2, 11], structural defects of peptide sequence and post-translational modifications

of OPN could also influence the inhibition of crystallization [13].

In conclusion, we have demonstrated that osteopontin and its mRNA were at distal and collecting ducts in rat kidney, and that their presence was enhanced in urinary stone-forming rats induced with oxalate precursor compared with normal rats. As it has showed inhibitory activity against calcium oxalate crystallization in vitro [28, 30], delivered to sites where urine is highly concentrated, and increased expression when exposed to a lithogenic challenge in vivo, so we can reasonably consider that OPN might play a significant role in normal defence against kidney stone formation.

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