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ORIGINAL PAPER

D. Asokan · P. Kalaiselvi · S. Muhammed Farooq
P. Varalakshmi

Calcium oxalate monohydrate binding protein: a diagnostic biomarker for calcium oxalate kidney stone formers

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Abstract Urinary oxalate is a biomarker for calcium oxalate kidney stone disease; however, its assay is insensitive and nonspecific. Calcium oxalate monohydrate (COM) binding protein (45 kDa) is a promoter of calcium oxalate kidney disease, which is markedly upregulated by oxalate induced oxidative stress. The current study was carried out to evaluate whether COM binding protein can serve as a diagnostic marker for calcium oxalate kidney stone formers. COM binding protein was isolated, purified and antibody was raised against it in rabbits. Urine samples (24 h) were collected from patients suffering from various kidney diseases such as acute nephritis, chronic nephritis, nephrotic syndrome, calcium oxalate (CaOx) stone formers, uric acid stone formers, struvite stone formers and calcium phosphate stone formers. This COM binding protein was quantified by an in house ELISA method and the excretion was found to lie between 2 and 3 mg in control samples, while in CaOx stone formers it was detected between 11 and 19 mg. Urinary risk factors were assayed. We conclude that COM binding protein can serve as a diagnostic marker for CaOx stone formers.

Keywords Calcium oxalate crystals · Oxalate binding protein · Oxalate · Calcium · Oxalate induced oxidative stress · Urolithiasis

Introduction

Urolithiasis has been cited as the third most common affliction of the urinary tract, characterized by an increased incidence and frequent recurrence of calcium oxalate (CaOx) stone in occidental societies [22]. In the currently existing medical management, urinary calculi can be easily removed or dissolved to remove the mineralized mass, but it is not possible to prevent their recurrence [3]. Preventive measures can be carried out if a diagnosis of stone formation is made at an early stage, however, to date no suitable marker for stone disease has been found. Urine excreted by normal individuals as well as by patients with CaOx stone is supersaturated with CaOx [6]. Therefore, supersaturation alone cannot be the sole cause for CaOx stone formation. The analysis of urinary electrolyte does not allow a discrimination between CaOx kidney stone patients and healthy individuals, and is not predictive of an individual's risk of forming stones. In order to evaluate the risk of stone formation, several other indices have been successively proposed, reflecting scientific progress in the elucidation of the process of urinary lithogenesis. However, their disparity shows the difficulty of attaining such an objective, and their frequent technical complexity limits their practical application. The diagnosis of 24 h urine samples cannot be a true representation of pathobiomineralization, as it is controlled by various factors including diet [14]. Hence, the scientific community has speculated on the possibility of various macromolecules influencing and directing the act of lithogenesis. This work is also an effort to identify a biomarker that will provide a clear picture of the oxalate induced oxidative stress condition.

D. Asokan · P. Kalaiselvi · S. Muhammed Farooq
P. Varalakshmi (✉)
Department of Medical Biochemistry,
Dr. ALM PGIBMS, University of Madras,
Taramani Campus, Chennai 600 113 India
E-mail: drvlakshmi@yahoo.com

Materials and methods

Diethylaminoethyl (DEAE) cellulose, Sephadex G 200 and dialysis sacs were obtained from Sigma (St. Louis, USA). Acrylamide, Triton X-100 and toluene were obtained from SISCO (India). Goat anti-rabbit IgG conjugated with horse radish peroxidase was obtained from the National Institute of Immunology, New Delhi, India. Human cadaveric kidneys were obtained from accident cases from the Forensic Science Department, Madras Medical College, Chennai, India. 24 h urine

samples(with sodium azide as the preservative) were collected from healthy volunteers as controls ($n = 68$, 40 males and 28 females) with a mean age of 48 ± 8 years. 24 h urine samples were collected from patients at Stanley Medical Hospital, Chennai as follows: CaOx stone formers ($n = 56$, 49 males and 7 females) with a mean age of 52 ± 7 years, recurrent CaOx stone formers ($n = 12$, 12 males) with a mean age of 56 ± 3 years, uric acid stone formers ($n = 18$, 12 males and 6 females) with a mean age of 50 ± 6 years, calcium phosphate stone formers ($n = 20$, 14 males and 6 females) with a mean age of 50 ± 9 years, struvite stone formers ($n = 9$, 2 males and 7 females) with a mean age of 49 ± 5 years, acute renal failure ($n = 18$, 9 males and 9 females) with a mean age of 60 ± 5 years, chronic renal failure ($n = 12$, 7 males and 5 females) with a mean age of 63 ± 5 years and nephrotic syndrome ($n = 16$, 10 males and 6 females) with a mean age of 60 ± 5 years. Urine was collected from the stone patients who had been admitted to urological clinics, and urine samples were collected prior to medication in the preoperative condition. All procedures were carried out as approved by the Ethics Committee of the University of Madras.

Isolation of COM crystal adsorbing protein

Human kidney was homogenized and a 10% solution, extracted with 1% TritonX-100, was incubated overnight and centrifuged at 17,500 *g* for 30 min. The supernatant obtained in this step was used for further experiments.

The adsorption system used for the isolation of the protein was a slightly modified procedure after Leal and Finlayson [12]. Calcium oxalate monohydrate (COM) crystals were prepared before use by mixing 1.5 M calcium chloride and 0.3 M potassium oxalate in the ratio of 1:5 (pH 6.5) by constant shaking at room temperature. After 30 min of stabilization of the system by agitation, protein from tissue homogenate and urine (~3 mg protein/mg crystal) was allowed to interact with the crystals with constant shaking of the solution for 1 h. The solution was centrifuged at 4,000 *g* for 10 min and the precipitated CaOx was washed with water three times to remove the extraneously bound protein. EDTA (25 mM) was used for the extraction of the bound protein. The EDTA extract was separated by centrifugation at 10,000 *g* at 4°C for 10 min and dialyzed against water at 4°C overnight with two changes of water.

Purification of COM binding protein

About 1.2 mg of protein was loaded onto a DEAE cellulose column, which was equilibrated with 0.05 M Tris-HCl buffer (pH 7). The elution was carried out using 0.05 M Tris HCl buffer (pH 7) followed by 0.05 M NaCl and finally 0.3 M NaCl in the same buffer. Twenty 2 ml fractions were collected in each buffer. These were then monitored in a UVIKON 930 spectrophotometer at 220 m .The partially purified protein was loaded onto a Sephadex G-200 column for chromatography (1×20 cm)and eluted with 0.01 M Tris-HCl buffer (pH 7).

Molecular weight determination

The purity and molecular weight of the protein was determined using the method of Laemmli [11].

Production of antibody

Purified COM binding protein from kidney in 2 ml of 1.5 mM NaCl was emulsified with an equal volume of complete Freund's adjuvant and injected intramuscularly with 500 μg of the protein at 24 sites on the back and then again at 2nd, 4th, and 6th week using incomplete Freund's adjuvant. The rabbit was bled 8 weeks after the initial immunization. Serum was collected by centrifugation at 4°C after clotting and stored at −80°C.

Enzyme linked immunosorbent assay

Urinary excretion of COM binding protein from the 24 h control and patient urine was quantified by enzyme linked immunosorbent assay (ELISA) [20].

Immunoblotting

Immunoblotting was carried out by the method of Towbin et al. [21]. Urine samples from patients with various kidney disorders were dissolved in SDS sample buffer. The samples were separated by 10% SDS PAGE under non-reducing conditions. They were then transferred to polyvinylidine difloride paper using a semidry electro blotting apparatus at 40 mA for 90 min at 23°C. The PVDF membrane was removed, and quenched with 2% BSA in phosphate buffered saline for 2 h to saturate additional binding sites. Blots were rinsed in PBS-T and incubated with 45 kDa polyclonal antibodies (1:500) for 1 h, washed three times with PBS-T and finally incubated with HRP-anti-rabbit IgG (1:500) for 1 h. The blots were washed with PBS-T and soaked in a substrate solution containing 8 mg of DAB until the bands were clearly visible. The reaction was terminated after 30 min by washing with water. The blots were protected from light and photographed.

Estimation of urinary risk factors

Urinary calcium was estimated by atomic spectrophotometry and oxalate [5], uric acid [2], creatinine [16]

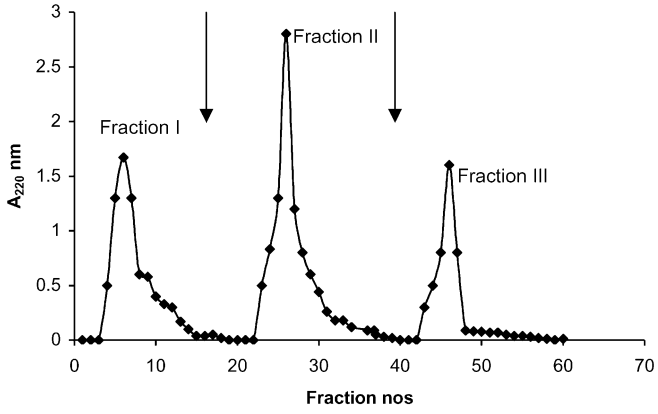

Fig. 1 DEAE cellulose elution profile of COM binding proteins isolated at pH 7.0

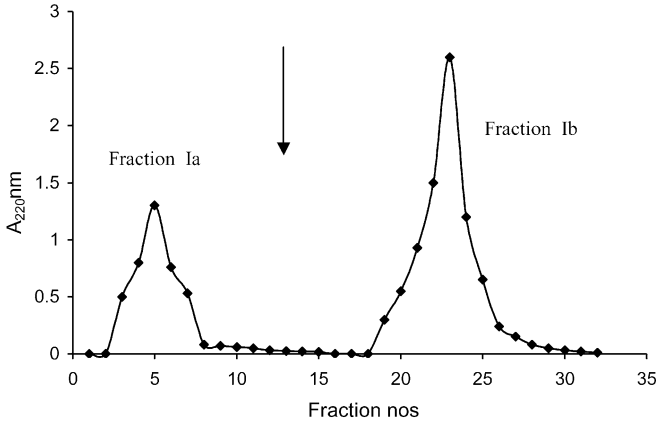

Fig. 2 Sephadex elution profile of fraction I isolated from DEAE cellulose chromatography

protein [13] and citric acid [17] were estimated by standard methods.

Results

DEAE cellulose column elution profile of human CaOx binding proteins isolated at pH 7.0 is shown in Fig. 1. The protein peak obtained in the Tris-HCl buffer 0.05 M (pH 7) was designated as fraction I, the peaks obtained in Tris-HCl buffer containing 0.05 M NaCl and 0.3 M NaCl were designated as fractions II and III, respectively (Fig. 1). The Sephadex elution profile of fraction I is shown in Fig. 2. Since fraction Ib promotes CaOx crystallization, we selected this for further studies [7, 19]. The molecular weight of fraction Ib was 45 kDa (Fig. 3). Western blot results revealed that this protein is excreted in urine in its native form (Fig. 4). Patients admitted to urological and nephrological clinics were thoroughly analysed by case histories, and the urinary risk factors were assessed. We found that in CaOx stone formers, calcium ($P < 0.001$), oxalate ($P < 0.001$), protein ($P < 0.001$) (protein excretion alone is relatively higher in

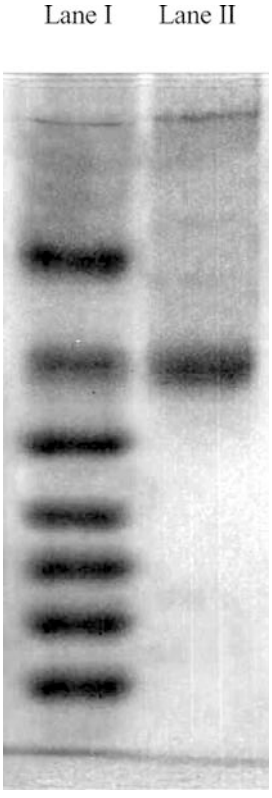

Fig. 3 SDS-PAGE of purified human fraction Ib COM binding protein. *Lane I* molecular weight markers (66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa and 14 kDa). *Lane II* 45 kDa COM binding protein from human cadaveric kidney

chronic nephritis, acute nephritis and nephrotic syndrome) and uric acid levels ($P < 0.001$) were significantly elevated when compared to the controls (Table 1). When the quantification of COM binding protein was performed, the level of 45 kDa protein was significantly ($P < 0.001$) higher than the controls only in the case of CaOx stone formers (Table 2). Normal urinary excretion of COM protein is found to lie between 2 and 3 mg. In CaOx stone formers, the range of this protein was found to lie between 11 and 19 mg. Its excretion was also elevated in recurrent stone formers. The elevation was more or less similar to that observed in patients with a first incidence of stones.

Discussion

CaOx urolithiasis, is a multifactorial process in which the nucleated crystals grow, aggregate and become attached to the renal cells and mature to form a stone. This act of stone formation is dictated by cellular and urinary macromolecules. The calcium and oxalate levels in control and hyperoxaluric urine suggest that other than mineral composition, organic macromolecules also determine lithogenesis. Numerous proteins, such as histone oxalate binding protein, mitochondrial oxalate binding protein and CaOx binding protein, which pro-

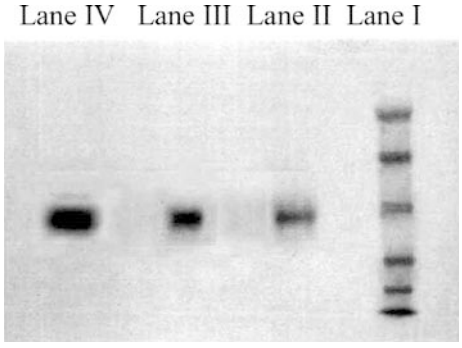

Fig. 4 Immunoblotting of human 45 kDa COM binding protein. *Lane I* molecular weight markers (121 kDa, 91 kDa, 46 kDa, 32 kDa, 24 kDa and 20 kDa). *Lane II* purified 45 kDa COM binding protein from human cadaveric kidney. *Lane III* COM binding protein (45 kDa) from control urine. *Lane IV* COM binding protein (45 kDa) from stone patient urine

Table 2 Quantification of urinary excretion of 45 kDa protein in controls, calcium oxalate stone formers and other kidney diseases. Values are expressed as mean ± SD of mg/24 h urine production. Values are statistically significant: $^{***}P < 0.001$, NS not significant. [a]compared with control, [b] compared with calcium oxalate stone formers. Normal range: 2–3 mg/24 h, stone formers: 11–19 mg/24 h. Sensitivity: 90%

	45 kDa COM binding protein
Normal ($n = 68$)	2.53 ± 0.5
Calcium oxalate stone formers ($n = 56$)	$15.37 \pm 4.3^{a***}$
Recurrent calcium oxalate stone formers ($n = 12$)	$16.45 \pm 3.25^{a***}$
Calcium phosphate stone formers ($n = 20$)	$3.46 \pm 1.63^{aNS\ b***}$
Uric acid stone formers ($n = 18$)	$3.5 \pm 1.3.83^{aNS\ b***}$
Struvite stone formers ($n = 9$)	$2.8 \pm 0.3^{aNS\ b***}$
Acute nephritis ($n = 18$)	$3.6 \pm 1.1^{aNS\ b***}$
Chronic nephritis ($n = 12$)	$3.5 \pm 1.2^{aNS\ b***}$
Nephrotic syndrome ($n = 16$)	$2.3 \pm 0.4^{aNS\ b***}$

mote CaOx crystallization, have been isolated from rat as well as human kidneys [19].

COM crystals are more commonly found in the urine of stone formers, while CaOx dihydrate (COD) is more frequently observed in asymptomatic crystalluria [18]. The formation of COD in preference to COM may be protective against stone disease by reducing attachment to renal tubules cells [23]. On the other hand, an oxalate load almost triples the risk of stone formation when compared to a similar calcium load [9]. Hence, the identification of macromolecules that are modulators can solve the problem of detecting and discriminating stone formers from non-stone formers. We have isolated a COM binding protein (45 kDa) which promotes CaOx crystallization [7, 19]. It is constituted of basic amino acids and is localized in the proximal and distal tubular region of the kidney. At the subcellular level, it is found to be present in the nucleus and mitochondria (data not shown). In the present investigation, we intended to check whether this protein could be used as a diagnostic marker for CaOx kidney stone formers.

The function of stone markers is to detect the presence of the diseases initially or at recurrence. In establishing COM binding protein (45 kDa) as a kidney stone marker it is necessary to define the normal range of COM binding protein in the urine of healthy subjects and compare this with the value in the urine of stone patients. The major finding of this clinical study is that the concentration of urinary COM binding protein is greater in CaOx kidney stone formers (11–19 mg) than in any other kidney disorder. There is no gender difference in the excretion of COM binding protein. The excretion of this protein was also monitored in other stone disorders where it was found to remain unaltered. Several other urinary proteins such as crystal matrix protein, nephrocalcin and bikunin are excreted in higher quantities in stone formers than in controls [8]. These proteins have not been exposed to vigorous clinical studies. Osteopontin excretion is higher in stone formers, but is elevated when the calcium level is increased. Factors reported to increase osteopontin expression also include exposure to bacterial infections, angiotensin II and renal ischemia [6].

We have shown that X-rays failed to detect CaOx stones in seven patients out of 56 cases. In these cases, COM binding protein excretion was between 5 and 7 mg, which is just below the threshold value for stone formation (11–19 mg). We therefore hypothesise that this value represents the initiation of stone formation. Patients presenting with renal colic often have a radio-

Table 1 Urinary excretion of calcium oxalate urinary risk factors in controls, calcium oxalate kidney stone formers and other kidney diseases. Values are expressed as mean ± SD of mg/24 h urine production. Statistically significant difference when compared to the control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS not significant

	Oxalate	Calcium	Citrate	Protein	Uric acid	Creatinine
Control ($n = 68$)	25.33 ± 5.8	196.28 ± 38	650.21 ± 110	92.16 ± 15.16	290.83 ± 60.43	820.11 ± 120.36
CaOx stone formers ($n = 56$)	$45.5 \pm 12^{**}$	$250.3 \pm 38^{*}$	$475.36 \pm 50^{**}$	$168.26 \pm 20.8^{*}$	$380.81 \pm 135^{**}$	$645.00 \pm 86.5^{*}$
Recurrent stone formers ($n = 12$)	$50.5 \pm 11.3^{**}$	$258.56 \pm 42.6^{*}$	$525.12 \pm 60.53^{**}$	$176.26 \pm 18.3^{*}$	$375.56 \pm 140^{*}$	$657.00 \pm 90.2^{*}$
Calcium phosphate stone formers ($n = 20$)	40.2 ± 18^{NS}	$260.34 \pm 36.2^{*}$	$510.78 \pm 55.21^{**}$	$158.17 \pm 21.13^{*}$	$350.75 \pm 120^{*}$	$620.00 \pm 101.9^{*}$
Uric acid stone formers ($n = 18$)	38.1 ± 15^{NS}	$246.26 \pm 31.2^{*}$	$525.29 \pm 58.26^{*}$	$161.36 \pm 22.13^{*}$	$450.36 \pm 50^{***}$	$592.00 \pm 96.21^{*}$
Struvite stone formers ($n = 9$)	26.3 ± 2.9^{NS}	$235.36 \pm 20.2^{*}$	530.36 ± 49.23^{NS}	$167.23 \pm 24.16^{*}$	320.29 ± 46^{NS}	$631.00 \pm 92.45^{*}$
Acute nephritis ($n = 18$)	20.2 ± 4.3^{NS}	$150.36 \pm 25^{*}$	670.23 ± 95.26^{NS}	$1{,}500 \pm 265.87^{***}$	$183.26 \pm 21^{**}$	$480.33 \pm 30^{*}$
Chronic nephritis ($n = 12$)	40.21 ± 11^{NS}	$128.29 \pm 22.3^{*}$	610.27 ± 158^{NS}	$1{,}980.86 \pm 1{,}281.16^{***}$	$168.31 \pm 21^{***}$	$420.43 \pm 36^{**}$
Nephrotic syndrome ($n = 16$)	23.5 ± 2.1	$148.23 \pm 10.5^{*}$	638.25 ± 55.7^{NS}	$1{,}451.18 \pm 143.6^{***}$	$180.27 \pm 27.3^{**}$	$501.29 \pm 52.3^{*}$

graph of their kidneys, ureter and bladder or an intravenous pyelogram (IVP) performed during the acute event. IVP is also useful in detecting the presence of radiolucent stone, ureteral calculi or obstruction. Patients undergoing IVP are frequently at a high risk for contrast induced nephropathy [10]. On the other hand, small calculi are easily detected by ultrasound image in a dilated collecting system. However, ultrasound fails to visualize most of the ureteric calculi [1, 15]. Through the use of the present biomarker, it is possible to detect the ureter stones.

In a routine clinical laboratory it is very difficult to assess the various urinary risk factors such as calcium, magnesium, phosphorus, uric acid and citrate which are time consuming, expensive and also inaccurate as they undergo daily fluctuations. Oxalate is a biomarker for CaOx kidney stone formers; however, in general, urinary oxalate is still assayed poorly. The reasons for this unreliability are multifactorial, but it is most frequently due to an inadequate work-up of the urine prior to the actual measurement, particularly in the case of urine samples that contain crystals of CaOx. Unless great care is taken to acidify the entire urine sample to a pH of 1.6 or to shake the whole urine thoroughly to disperse the crystals homogeneously before sub-aliquoting, the probability of over or underestimating oxalate is extremely high [4]. There is no clear-cut demarcation in urinary oxalate levels between stone formers and non-stone formers. Crystalluria is also seen in non-stone formers [6]. Hence, urinary oxalate or calcium levels alone cannot be an index for stone recurrence. In addition, the excretion of COM binding protein has been monitored in rats fed with soya, which has both oxalate and antioxidants, and the excretion of this protein is unaltered although the rats exhibited hyperoxaluria. Further, the analysis of soya fed rats revealed the absence of CaOx crystals in urothelium due to soya's antioxidant effects, overwhelming the oxalate content (D. Asokan unpublished data) which clearly indicates that this protein is expressed only during oxalate induced oxidative stress conditions, i.e. during CaOx stone formation.

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