Role of a protein inhibitor isolated from human renal stone matrix in urolithiasis

S Aggarwal, C Tandon¹, M Forouzandeh, S K Singla, R Kiran and R K Jethi*

Department of Biochemistry, Panjab University, Chandigarh-160 014, India
¹Jaypee University of Information Technology, Waknaghat, Solan (H.P.)

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The role of biomolecule(s) from renal stone matrix in urolithiasis was investigated. The ability of a particular fraction (>10 kDa fraction) isolated from the EDTA extract of powdered human renal stones to influence calcium oxalate monohydrate (COM) crystal growth was studied. The most potent inhibitor of COM crystal growth obtained from >10 kDa fraction was purified by various chromatographic techniques and SDS-PAGE, etc. and was found to have a molecular mass of 36 kDa. The urine and serum samples obtained from normal persons were found to be more potent in inhibiting the growth of COM crystals as compared to the kidney-stone patients. Polyclonal antibodies were raised against this inhibitor and were employed to determine the concentration of 36 kDa inhibitor in urine and serum samples of normal persons and kidney-stone patients.

Keywords: Kidney, calculi, calcium oxalate, organic matrix, inhibitor.

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Human urine contains stone-forming constituents and has potential to induce spontaneous mineralization¹. Urolithiasis, the deposition of stones in urinary tract, is a complex, multifactorial disease resulting from the interaction between environmental and genetic factors and continues to pose a universal health problem. Although, the calculi can be lodged in any part of the urinary tract, occurrence in the upper urinary tract (urinary calculosis) is common. The main chemical constituents in urinary stone may include calcium oxalate monohydrate, calcium oxalate dihydrate, hydroxyapatite, carbonate apatite, octacalcium phosphate and brushite². Calcium oxalate monohydrate (COM) is reported to initiate mineralization, followed by deposition of calcium oxalate dihydrate (COD)³. Besides, urinary stones also contain uric acid, sodium/ammonium urate, magnesium/ammonium phosphate (apatite and struvites) and cystine⁴. Although human urine is supersaturated with stone forming constituents, it also contains constituents which inhibit crystal growth, nucleation, aggregation and cell attachment of crystals⁵-⁷. In addition, dietary and urinary oxalate is known to play an important role in urinary stone formation⁸. It is also reported that the saturation state of body fluids with respect to stone-forming constituents and the presence of various biomolecules (inhibitors/stimulators) in the body fluids as well as organic matrix are known to influence mineralization. The matrix displays a variable and complex composition and a few proteins of matrix are common in various stones. It may act as a catalyst to induce mineral phase formation from metastable solutions. The biomolecules asso-ciated with the matrix may control mineralization by acting as inhibitors/stimulators⁷-¹².

Although improved non-surgical techniques, such as extracorporeal shock wave lithotripsy, transurethral lithotripsy, ureteroscopy and percutaneous nephrolithotomy have been used in the management of urolithiasis successfully, its high recurrence still poses a serious problem¹³-¹⁵. Therefore, understanding the etiology of renal calculosis at the molecular level could lead to its better management. Earlier studies though found an organic matrix (uromucoid or glycoprotein) associated with most of the urinary calculi, but its role in calculosis is not clearly understood⁵,¹²,¹⁶-²⁵. In the last two decades, the main thrust of urolithiasis research has been on identification and characterization of biomolecules present in the body fluids which are capable of retarding the onset of nucleation and the subsequent growth of calcium oxalate hydrates and calcium phosphate crystals, commonly found in renal calculi⁵,¹⁹,²⁰,²⁶.

In the present study, attempts have been made (i) to understand in vitro mineralization of renal calculi (stones in renal pelvis) by an assay system involving precipitation of calcium and oxalate as mineral phase; (ii) to isolate and purify biomolecule(s) from the renal calculi extract capable of influencing in vitro mineralization; and (iii) to develope ELISA-based assay system employing polyclonal antibodies (PCA) against such potent biomolecule(s).
Materials and Methods

Clinically normal persons (10) having no previous history of either urolithiasis or any other disorder of mineral metabolism served as control group. Patients (50) suffering from no other abnormality but for non-recurrent and non-infectious stones at the Urology Department of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh served as experimental group. Kidney stones were detected by abdominal X-ray of the patients and confirmed by intravenous pyelography. Urine and serum samples were collected from persons aged >25 yr from both the groups. The patients and normal subjects were advised to void their bladder at 8 A.M. and thereafter their urine samples were collected in plastic bottles (containing a few crystals of thymol as preservative) until 8 A.M. the following morning. The samples were chemically analysed and preserved at 4°C.

Renal calculi and demineralization of stones

Human renal calculi (50), surgically removed from kidney stone patients were obtained from the Urology Department, PGIMER, Chandigarh and analysed by standard biochemical methods. Of these, 30 stone samples with calcium oxalate as the major component were randomly pooled into 5 groups, each group having 6 samples.

The samples were demineralized by modified method of Dussol et al\(^{21}\). The selected stones were washed in 1.5×10\(^{-1}\) M NaCl with gentle stirring for 48 hr and then pulverized to a fine powder and extracted with a solution containing 5.0×10\(^{-2}\) M EDTA, 1.0×10\(^{-3}\) M PMSF and 1% β-mercaptoethanol for 4 days at 4°C. The suspensions and supernatants obtained were filtered through an Amicon Model 200 apparatus (cut off mol. mass 10 kDa) under nitrogen at 40 p.s.i., concentrated and the volume was made up to 10 ml with distilled water.

COM-crystal growth assay

The potency of the urine and serum samples to influence COM crystal growth was assayed as reported\(^{27}\). The inhibitory activity was calculated using the equation\(^{28}\):

\[
*\text{Inhibitory activity} = \frac{C_i - C_w}{C_i - C_t} \times \frac{C_i - C_{\infty}}{C_w - C_{\infty}} \quad \ldots (1)
\]

where \(C_i = ^{14}\text{C cpm} \) at zero time, \(C_w \) and \(C_t = ^{14}\text{C cpm} \) at 40 min in the absence and presence of sample, respectively and \(C_{\infty} = ^{14}\text{C cpm} \) at 24 hr.

COM crystal growth assay was standardized under our laboratory conditions\(^{19,27,28}\). The decrease in \(^{14}\text{C}-\)oxalate concentration in solution at different time intervals after addition of seed crystals was taken as an index of the rate of COM crystal growth in a metastable solution of calcium chloride (CaCl\(_2\).2H\(_2\)O) and sodium oxalate\(^{26}\).

Purification of potent biomolecule(s)

DEAE-cellulose (anion-exchanger), molecular sieve chromatography and SDS-PAGE were employed to purify potent biomolecule(s) from renal calculi extract (EDTA) using a linear NaCl gradient (5.0×10\(^{-2}\)-5.0×10\(^{-1}\) M) 5.0×10\(^{-2}\) M Tris-HCl buffer, pH 7.4 (Fig. 1A). The biomolecule responsible for COM crystal growth inhibitory activity was further purified by loading the respective fraction (pooled fraction VII of DEAE-cellulose of >10 kDa fraction) on Sephadex G-75 column, pre-washed with 5.0×10\(^{-2}\) M Tris-HCl buffer (pH 7.4) and 5.0×10\(^{-2}\) M NaCl and 140 fractions of 3 ml each were collected and absorbance was recorded at 280 nm.

Raising of polyclonal antibodies (PCA)

PCA were raised against the most potent purified biomolecule, influencing calcium oxalate precipitation. The concentration of the inhibitory biomolecule in the urine and serum samples obtained from normal persons and kidney stone patients was determined by ELISA method using PCA. The concentration of Ca\(^{2+}\), Mg\(^{2+}\), phosphate, oxalate, and citrate ions and creatinine in urine and blood samples were estimated by methods described earlier\(^{29,34}\).

The data were analysed by Student’s \(t\)-test.

Results and Discussion

The whole EDTA renal calculi extract showed inhibitory activity of 250±11.8 units per 100 ml. The >10 kDa fraction of the EDTA extract which exhibited 72% of the total inhibitory activity, i.e., 180.1±9 units per 100 ml was employed for subsequent isolation and purification of potent inhibitory biomolecule. DEAE-cellulose (anion-exchange) chromatography of >10 kDa fraction (Fig. 1B) indicated two types of biomolecules (inhibitors and stimulators) responsible for influencing the growth of COM crystals. The pooled fraction VII of DEAE-cellulose column exhibited maximum inhibitory activity (919.2±3 units/100 ml), followed by fractions X (437.2±25) and IX (53±7), while the pooled fraction IV showed maximum
stimulatory activity (89±5 units/100 ml), followed by fractions II (17±0.5) and I (15.0±0.1).

The rate of disappearance of \(^{14}\)C-labelled oxalate \((k)\) from the solution containing seed crystals was significantly influenced by >10 kDa fraction (Fig. 2A). Higher counts observed at all the time intervals (less ion uptake or growth) in the presence of fraction VII confirmed the inhibitory nature of the fraction (Fig. 2B).

The values of \(k\) calculated by plotting \((\text{Ox})_t - (\text{Ox})_\infty\) vs ‘t’ for control and >10 kDa EDTA fractions of renal calculi extract (Fig. 3A) were 71.11±2.12 and 64±1.12 M\(^{-1}\) min\(^{-1}\) (mg/100 ml)\(^{-1}\), respectively. The COM crystal growth rate constant \((k)\) in the absence and presence of most potent inhibitory DEAE-cellulose fraction VII was 45±1.81 and 25.26±2.05 M\(^{-1}\) min\(^{-1}\) (mg/100 ml)\(^{-1}\), respectively (Fig. 3B). The kinetics of COM crystal growth are of second order and can be represented by the loss of Ca\(^{2+}\) or oxalate ions \((\text{Ox})\) from the aqueous medium by the following equation\(^{28}\):

\[
\frac{d[\text{Ca}]}{dt} - \frac{d[\text{Ox}]}{dt} = k[(\text{Ca})_t - (\text{Ca})_\infty]^2 + k[(\text{Ox})_t - (\text{Ox})_\infty]^2 \quad \ldots (2)
\]

where \((\text{Ca})_t\) and \((\text{Ox})_t\) are calcium and oxalate concentrations at time ‘t’, \((\text{Ca})_\infty\) and \((\text{Ox})_\infty\) are concentrations at ‘equilibrium’ and ‘\(k\)’ is the rate constant.

The above equation can be integrated to give:

\[
[(\text{Ox})_t - (\text{Ox})_\infty] = kt + [(\text{Ox})_\infty - (\text{Ox})_0] \quad \ldots (3)
\]

where \((\text{Ox})_0\), \((\text{Ox})_t\) and \((\text{Ox})_\infty\) are oxalate concentrations at zero, \(t\) and equilibrium time, respectively. The plot of \([((\text{Ox})_t - (\text{Ox})_\infty)]\) vs ‘\(t\)’ therefore, should give a straight line with the
empirical COM seed crystal growth rate constant $k$ in $M^{-1} \text{min}^{-1}$ (mg/100 ml)$^{-1}$ obtained from the slope. The magnitude of $k$ in the present study was observed to be directly proportional to the crystal growth$^{10}$.

The most potent DEAE-cellulose fraction VII was further purified on Sephadex G-75 column and the two major fractions $F'_1$ and $F'_2$ were obtained (Fig. 4). Inhibitory activity of the two G-75 pooled fractions $F'_1$ and $F'_2$ to influence COM crystal growth was found to be 91.2±20.4, and 730.5±40 units/100 ml, respectively. SDS-PAGE of fraction $F'_2$ indicated that the inhibitory activity was due to the presence of a single biomolecule having molecular mass of approx. 36 kDa (Fig. 5).

The present study demonstrated that urine and serum samples from normal persons as well as kidney-stone patients contain biomolecules capable of inhibiting COM crystal growth. The inhibitory activity was 4.23±1, 2.50±0.53, and 0.41±0.05, 1.64±0.41/100 ml, respectively. Urine and serum samples from normal persons were found more potent compared to kidney-stone patients which was evident by significantly lower concentration of such biomolecules in samples from kidney-stone patients. However, these differences in the inhibitory potency could not be observed in ELISA-based estimation employing PCA raised against 36 kDa protein inhibitor.

Earlier, we reported a significantly higher concentration of a 66 kDa stimulator of COM crystal growth in the stone matrix in the urine and serum samples obtained from kidney-stone patients, compared to normal persons$^{26}$. In addition, lower amount of a glycoprotein inhibitor, nephrocalcin (14 kDa) of calcium oxalate crystallization was found in the urine of kidney-stone patients, compared to normal persons$^{20}$. Human urine is also known to contain other proteins, such as Tamm-Horsfall protein, crystal matrix protein (f1 prothrombin fragment), uronic acid-rich protein and glycosylated

\[ k \text{ in } M^{-1} \text{min}^{-1} (\text{mg/100 ml})^{-1} \]
phosphoprotein (osteopontin) which may also play an important role in the etiology of renal calculosis. The above observations assume practical significance in understanding the molecular events leading to stone formation. It is likely that under physiological conditions, a delicate balance exists between the concentrations/activities of various inhibitors and stimulators of mineral phase formation in the body fluids and its disturbance under specific pathological conditions may form an important element in the etiology of urolithiasis.

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