Urol Res (1996) 24:45–50

ORIGINAL PAPER

F. Atmani · F. J. Opalko · S. R. Khan

Association of urinary macromolecules with calcium oxalate crystals induced in vitro in normal human and rat urine

Received: 9 June 1995 / Accepted: 15 August 1995

Abstract This study was undertaken to identify proteins which are found associated with calcium oxalate crystals induced in vitro in normal human and rat urine. Crystallization was initiated by adding sodium oxalate individually to each urine sample without centrifugation and filtration. Crystals were collected and analyzed by scanning electron microscopy and X-ray diffraction. Crystal matrix proteins (CMPs) were obtained by demineralization of the crystals with ethylenediaminetetra-acetic acid (EDTA) and analyzed by western blotting technique for immunological identification. Crystals produced in human urine were found to be a mixture of calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) while those produced in rat urine were exclusively COD. CMPs extracted from crystals in human urine comprised, in addition to prothrombin-related proteins, osteopontin and albumin. However, CMPs extracted from crystals in rat urine contained only osteopontin and albumin. Prothrombin-related proteins were found only in trace amounts. In a separate experiment, rat urine samples were supplemented with COM before inducing crystallization. Similar results were observed showing that CMP contained osteopontin, albumin and trace amounts of prothrombin-related proteins. We conclude that several urinary macromolecules including not only prothrombin-related proteins, but also osteopontin and albumin, become associated with CaOx crystals. The incorporation of these proteins in growing stones is not only due to the presence of γ-carboxyglutamic acid as it was suggested for prothrombin-related proteins, but may be due to other factors such as urinary chemistry, presence of glutamic and aspartic acid residues, and calcium-binding sites.

F. Atmani (✉) · F. J. Opalko · S. R. Khan
University of Florida, College of Medicine, Department of Pathology and Laboratory Medicine, Gainesville, FL 32610-0275, USA, Fax: (+1) 904 392 6249

Key words Nephrolithiasis · Urine · Calcium oxalate · Prothrombin · Osteopontin · Albumin

Introduction

Regardless of the mineral composition, urinary stones contain about 2–5% organic matrix [2, 24, 29], of which two-thirds is protein [1]. This raises the possibility that the matrix proteins play a significant role in stone genesis. Therefore, extraction and identification of the urinary stone matrix proteins was a major topic of research for several workers. For Finlayson et al. [6] and Vermeulen et al. [28], presence of the organic matrix is simply a coincidence. Nevertheless, Leal and Finlayson [15] demonstrated that physical adsorption can account for only a part of the matrix protein. Khan and Hackett [12] have shown that the organic matrix is ubiquitous in urinary stones, and part of it appears to be incorporated within the crystals. In an in vivo experimental model, the same authors have demonstrated that the organic material becomes intimately associated with crystals during early stages of their formation [11]. One recent approach that has been adopted is to identify proteins extracted from calcium oxalate (CaOx) crystals generated in human urine in vitro [20, 21]. The major finding of these studies was that the incorporation of proteins into crystals is a selective phenomenon. Tamm-Horsfall protein (THP) and albumin, the most abundant proteins found in urine, were not detected in the crystal matrix. It was demonstrated that the major protein associated with urinary crystals had a molecular weight of 31 kDa and was named calcium matrix protein [3]. This protein was later shown to be related to prothrombin [26, 27] and it was identified as fragment F1 of prothrombin [25]. Nevertheless, in these earlier studies, human urine samples were centrifuged and filtered before inducing crystallization. We believe that these procedures contribute

to the loss of a significant portion of the urinary macromolecules and experiments should be conducted in whole native urine. Accordingly, we conducted the experiments in whole normal human urine and whole rat urine as well.

The objective of this study is to identify various protein components of the crystal matrix obtained from calcium oxalate crystals produced in vitro in whole human and rat urine without centrifugation and filtration.

Materials and methods

Individual 24-h urine specimens were collected in polyethylene bottles containing sodium azide from healthy human subjects with no urological or nephrological problem. Samples were refrigerated for the duration of collection. Rat urine was collected daily using metabolic cages from male Sprague-Dawley rats (Harlan Sprague-Dawley, Ind., USA) fed a normal diet. During the collection, urine receptacles containing two drops of 20% sodium azide were surrounded with dry ice.

CaOx crystals were induced in seven separate whole human urine samples by adding sodium oxalate at 37 °C in a shaker water bath as described earlier [3]. After 3 h, the crystals were collected by centrifugation at 10000 *g* for 30 min at room temperature in a J2-21 centrifuge (Beckman Instrument, Calif., USA). Crystals were washed with distilled water or 0.1 M sodium hydroxide solution for eight cycles, respectively. Then they were analyzed by scanning electron microscopy (SEM) and X-ray diffraction. Similar experiments were conducted in three rat urine samples. In addition, two rat urine samples were supplemented with COM crystals (BDH, UK) before inducing crystallization by adding sodium oxalate. In order to determine the effect of centrifugation on urinary proteins, some human urine samples were centrifuged at 10000 *g* for 30 min and the residue obtained was subjected to electrophoretic analysis.

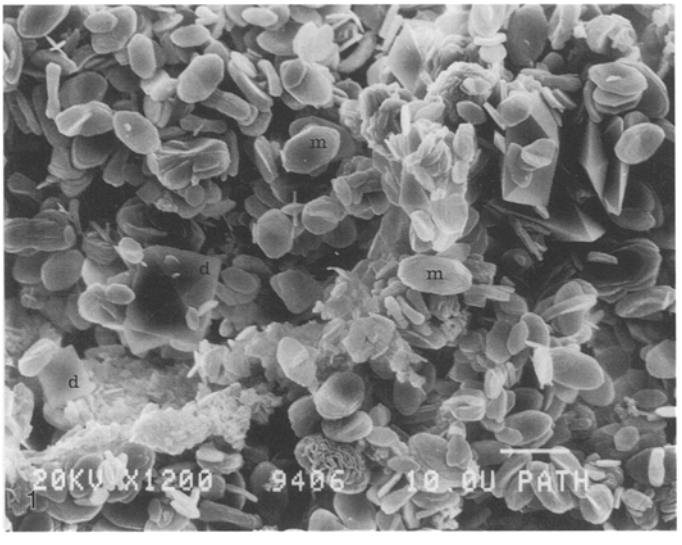
The crystals obtained from each urine sample were demineralized with 0.25 M ethylenediamine tetra-acetic acid (EDTA) at pH 8 for 3 days at 4 °C with stirring. Samples were dialyzed against demineralized water for 24 h at 4 °C using a dialysis membrane cut off 6–8 kDa (Spectrum Medical Industries, Calif., USA). They were lyophilized and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean II apparatus (Bio Rad Laboratories, Calif., USA). Gels were stained with Coomassie blue R-250 or using silver staining procedure [19]. Also, other gels were transferred onto a nitrocellulose membrane (Costar, Mass., USA) for immunological identification. The polyclonal antibodies used at a dilution of 1:1000 were as follows: prothrombin and albumin (ICS Biomedical, Calif., USA), α_1-microglobulin (Binding Site, Calif., USA), inter-α-trypsin inhibitor (ITI) (Accurate Chemical Scientific, N.Y., USA). This latter antibody was used to detect the light chain of ITI found in the urine. THP antibody was made in our laboratory in rabbit by injecting rat THP as antigen (Kel Farm Laboratory, Fla., USA) and osteopontin antibody was a gift from Dr. E. M. Worcester (Department of Medicine, Medical College of Wisconsin, Milwaukee, USA). The second antibody used at a dilution of 1:5000 and marked with alkaline phosphatase was from Hyclone (Hyclone Laboratories, Utah, USA). Reagents for immunodetection were from Bio Rad and all the other reagents were obtained from Fisher (Fisher Scientific, Pa., USA).

Results

Crystals induced in human urine were identified by SEM as a mixture of COM and COD (Fig. 1). However, the crystals generated in rat urine were exclusively COD (Fig. 2). X-ray diffraction confirmed these results. Figure 3 shows human urinary and crystal matrix proteins analyzed by SDS-PAGE (lanes 1–4), stained with Coomassie blue and the corresponding western blotting (lanes 5–8) immunostained with

Fig. 1 Scanning electron micrograph of calcium oxalate crystals induced in whole normal human urine. Dipyramidal calcium oxalate dihydrate crystals (*d*) are scattered among monoclinic calcium oxalate monohydrate crystals (*m*)

Fig. 2 Scanning electron micrograph of calcium oxalate crystals generated in whole normal rat urine. Only dipyramidal calcium oxalate dihydrate crystals are present



different antibodies. To determine the effect of centrifugation on urinary proteins, some human urine samples were centrifuged and the residue obtained was submitted to electrophoretic analysis as shown in lane 2 (Fig. 3). Several bands were observed, among them we identified THP, osteopontin and albumin by using specific antibodies on western blotting (not shown). The results demonstrate that centrifugation of urine contributes to the loss of a portion of proteins. Therefore, this procedure was avoided in all subsequent experiments.

Proteins extracted by demineralization of CaOx crystals generated in whole human urine by adding sodium oxalate and washed with distilled water showed the presence of four major bands with molecular weights (MWs) of about 31, 50, 67 and 95 kDa (lane 3). In order to identify these bands, different antibodies were used on western blotting and the results were as follows. We detected two bands at a MW of 31 and 45 kDa when prothrombin antibody was used (lane 5). In some samples, the band at 78 kDa was also detected. Osteopontin (lane 6) and albumin (lane 7) were detected in the same position at 67 kDa. Tamm-Horsfall protein (THP) was detected at 95 kDa (lane 8). Trace amounts of the light chain of ITI at 35 kDa and α_1-microglobulin (α_1-m) at 31 kDa were also detected (results not shown). When the crystals were washed with NaOH solution, only two major bands were observed, one with an MW of 31 and another with an MW of 67 kDa (lane 4). On western blotting we detected prothrombin-related proteins similar to those shown in lane 5, osteopontin similar to that in lane 6 and a small amount of albumin. THP, α_1-m and the light chain of ITI were not detected. It is important to point out that due to the high sensitivity of the western blotting technique, a band at 45 kDa which was not visible by Coomassie blue was detected when prothrombin antibody was used.

Figure 4 illustrates the combination of SDS-polyacrylamide gel (lanes 1–3) and western blot analysis (lanes 4–9) of CMP obtained from calcium oxalate crystals generated in whole rat urine. Lanes 2, 4, 6 and 8 show proteins extracted from crystals generated by sodium oxalate addition only and lanes 3, 5, 7 and 9 show proteins obtained from crystals generated by addition of sodium oxalate and COM crystals to the rat urine. More proteins were found in the CMP when calcium oxalate crystals were generated by addition of sodium oxalate in the urine supplemented with COM crystals (lane 2 vs 3). Minor amounts of prothrombin-related proteins were detected at 31, 45 and 50 kDa (lanes 4 and 5). Osteopontin and albumin were detected at approximately 70 kDa (lanes 6–9). Minor amounts of THP, α_1-m and the light chain of ITI were detected (results not shown). CMP obtained from crystals after washing with sodium hydroxide solution contained only osteopontin, albumin and trace amounts of prothrombin-related proteins.

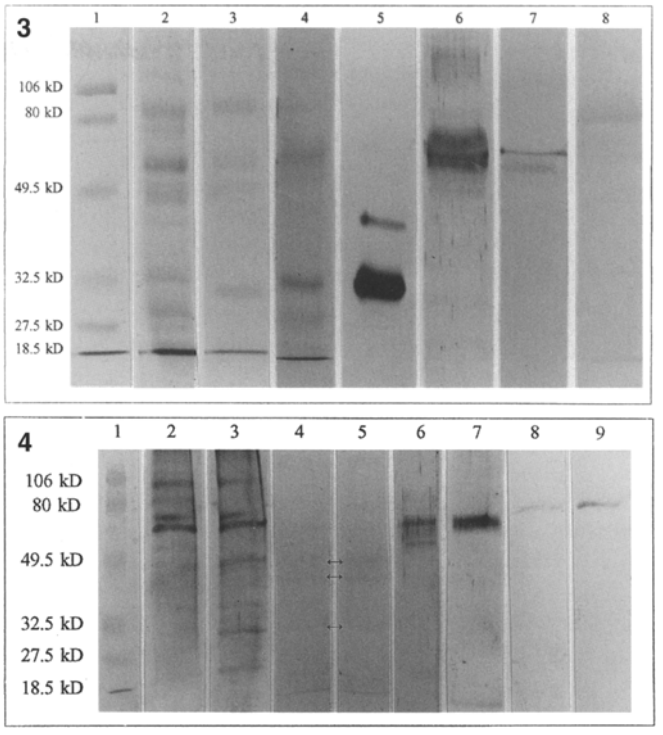


Fig. 3 SDS-polyacrylamide gel electrophoresis (*lanes 1–4*) and immunodetection on western blot (*lanes 5–8*). *Lane 1* molecular weight markers, *lane 2* residual proteins obtained after centrifugation of the whole human urine, *lane 3* crystal matrix protein obtained from crystals washed with distilled water, *lane 4* crystal matrix protein obtained from crystals washed with 0.1 M sodium hydroxide solution, *lane 5* prothrombin, *lane 6* osteopontin, *lane 7* albumin, *lane 8* Tamm-Horsfall protein

Fig. 4 SDS-polyacrylamide gel electrophoresis (*lanes 1–3*) and immunodetection on western blot (*lanes 4–9*): *lane 1* molecular weight markers, *lane* 2 crystal matrix protein obtained from crystals induced in rat urine, *lane 3* crystal matrix protein obtained from crystals induced in rat urine supplemented with COM crystals, *lanes 4, 5* prothrombin, *lanes 6, 7* osteopontin, *lanes 8, 9* albumin. In lanes *2, 4, 6 and 8* calcium matrix protein obtained from crystals is generated by adding sodium oxalate only. In *lanes 3, 5, 7 and 9* calcium matrix protein is obtained from crystals generated by adding sodium oxalate and calcium oxalate monohydrate crystals

Discussion

The presence of organic material in renal calculi has received considerable attention for many years. However, the role of this matrix as an inhibitor or a promotor of stone formation has not been definitely established. In either case, the mechanism involved in the adsorption of urinary macromolecules to crystals and stones remains unsolved. For this reason many researchers are involved in extracting and identifying various components of organic matrix of urinary stones. According to Boyce [1], 64% of this organic matrix is protein. Association of organic matrix with CaOx crystals starts very early during nephrolithiasis

[11]. Accordingly, in order to understand this interaction between organic material and crystals, it was suggested to induce crystallization in human urine in vitro and analyze the proteins extracted from the newly formed crystals [20, 21]. Incorporation of proteins into crystals was demonstrated to be a selective phenomenon. A single protein called crystal matrix protein was demonstrated to be selectively associated with CaOx crystals [4]. This protein was shown to be related to prothrombin [26, 27] and later identified as fragment F1 of prothrombin [25]. It was concluded that the presence of γ-carboxyglutamic acid (Gla) in the structure of prothrombin-related proteins, which has a high affinity for Ca^{2+}, may be the key to explain the association of this protein with CaOx crystals.

It is important to point out that the terms "incorporation" and "inclusion" have been used in previous studies to define the association of the organic material with CaOx crystals. In fact, these terms have led to a confusion as to how such macromolecules with a large molecular weight can be incorporated or included within crystals. Obviously, large molecules cannot be included in the atomic lattice of the crystal. It is more likely that the organic material bind ionically or by Van der Waals forces to the crystals on the surface depending on the proteins and the surrounding solution chemistry. It is probable that proteins are the adhesive material that bind crystallites in the earlier stages of crystal growth. These macromolecules are then forced out as mineralization occurs between crystals during calculi formation, which has been well documented in a previous study [29]. In addition, some strongly bound proteins may not be displaced by mineralization but remain between crystallites and become a part of the stone [12].

In our experiments, crystals generated in whole human urine were mostly COM. In previous study of Morse and Resnick [20, 21], the crystals obtained were also confirmed to be COM. However, a similar experiment conducted by Doyle et al. [4] showed that crystals induced in human urine were mostly COD. We think that perhaps normal urines in these studies had a different urinary chemistry.

In all the previous studies, crystals were generated in centrifuged and filtered human urine. Doyle et al. [3] have stated that centrifugation and filtration of the urine before crystallization had little effect on the quantitative protein content of the crystal extract even when they noticed that these procedures contributed to a complete loss of THP and to reduce the concentration of other proteins. Our results confirmed this reduction in a portion of the urinary proteins after urine centrifugation. For this reason we conducted our experiments in whole native urine without centrifugation and filtration.

Our results indicate that not only prothrombin-related proteins, but also albumin and particularly osteopontin, are associated with CaOx crystals formed in human urine. The presence of these proteins in the crystal matrix as described here and their reported absence in earlier studies, especially for albumin, is apparently due to our use of whole urine without centrifugation and filtration.

When the crystals were washed with distilled water, we also found THP, trace amounts of the light chain of ITI and α_1-m. This indicates that perhaps these macromolecules were not tightly bound to the crystals and they were removed easily by using strong agent (NaOH) for washing the crystals. It becomes therefore clear that the adsorption of proteins onto crystals is not totally a selective phenomenon as presented by previous investigators [3, 20, 21]. The pre-treatment of urine and the washing procedure may decide which proteins could be found in the organic matrix of the crystals.

The crystals generated in whole rat urine were found to be only COD. Proteins extracted from them were composed mainly of osteopontin and albumin. Prothrombin-related proteins were found only in trace amounts. Since rat urine produced only COD crystals, we hypothesized that fragments of prothrombin may be adsorbed selectively to COM crystals. To clarify this point, we supplemented two separate rat urine samples with COM crystals before inducing crystallization. However, the results remained unchanged, showing the presence of osteopontin, albumin and low amounts of prothrombin-related proteins. We have checked for the presence of prothrombin-related proteins in human and rat urine and they were found in both (results not shown). Thus there appears to be a major difference between human and rat urine in terms of the association of urinary macromolecules with CaOx crystals. It was postulated that prothrombin-related proteins were selectively incorporated into CaOx crystals, and this inclusion is due to the presence of Gla residues [3]. Lian et al. [17] isolated a Gla-rich protein from kidney stones and pointed out the importance of Gla residues in binding to calcium. Nevertheless, our results showed that even though prothrombin-related proteins are present in the rat urine, they are present only in a trace amount in CaOx crystals produced in rat urine.

Osteopontin, which is a potent inhibitor of crystallization [23, 31] and does not contain Gla, was found in matrices of CaOx crystals and kidney stones [10, 14]. Electron microscopic studies have also shown the presence of this protein in the matrix of CaOx stones induced in rats in vivo [18]. Since osteopontin contains about 50% of glutamic and aspartic acid residues [8], these amino acids may have a role in the inhibitory activity of urinary macromolecules as proposed by Kohri et al. [13] and may also participate in crystal-protein interaction. Furthermore, the presence of nine consecutive aspartic acids in the structure of osteopontin may explain its high affinity for calcium.

Albumin has also been found in kidney stones. Indeed, Fraij [7] has demonstrated that THP, transferrin

and albumin accounted for more than 40% of the total matrix protein of urinary stones. Worcester et al. [30] have also shown that albumin has a strong affinity for COM crystals. In addition, Dussol et al. [5] have analyzed the protein extract from five different morphological types of stones and have found that albumin was the most abundant protein. The most interesting aspect of the Dussol et al. [5] study is the demonstration that proteins found in the organic matrix of stones are in fact bound to human serum albumin, rather than directly binding to the crystals.

The adsorption of THP to kidney stones remains a subject of debate. It seems that the association of THP with stones depends on the physicochemical conditions of the urine. Accordingly, we suggest that the association of macromolecules with crystals is not due only to the presence of Gla or glutamic and aspartic acid, but probably due also to the urinary chemistry of the urine. Indeed, it is known that chemistry of rat urine is different from that of the human [16, 22]. The concentration of anionic or cationic substances may affect the conformational structure of urinary proteins and thereby modulate their physiological activity. Such modification was revealed by Hess [9], who demonstrated that the presence of citrate, which is slightly inhibitory, reversed the promoting effect of THP into aggregation inhibition.

It should however be recognized that the conditions in which crystallization was carried out in our study and in other similar studies may be very different from the conditions present in renal tubules, where crystals form during nephrolithiasis. Thus the results cannot be directly extrapolated to the in vivo situation. The high concentration of oxalate used to induce CaOx crystals formation in vitro may be possible only in primary hyperoxaluria patients and never be found in urine of idiopathic stone formers. The impact of such high levels of oxalate on the structure and function of various urinary proteins and their crystal binding behaviour is currently unknown. Additionally, calcium oxalate crystals formed in excess oxalate may have different surface charge than the crystals formed in the presence of excess calcium and thus may have different binding characteristics.

We conclude that inclusion of urinary macromolecules in crystal matrix may not be as selective a process as previously reported. Prothrombin-related proteins are not the only constituent of crystal matrix but osteopontin and albumin are also associated with CaOx crystals. The incorporation of urinary proteins in the crystals may not only be due to Gla residues or glutamic and aspartic acids, but other factors such as urinary chemistry may also be involved. The presence of these proteins in crystal matrix indicates the possibility that these proteins may be involved in modulating crystallization either individually or in concert with each other to give a resultant effect.

References

1. Boyce WH (1968) Organic matrix of human urinary concretions. Am J Med 45:673
2. Boyce WH, Garvey FK (1956) The amount and nature of the organic matrix in urinary calculi: a review. J Urol 76:213
3. Doyle IR, Ryall RL, Marshall VR (1991) Inclusion of proteins into calcium oxalate crystals precipitated from human urine: a highly selective phenomenon. Clin Chem 37:1589
4. Doyle IR, Marshall VR, Dawson CJ, Ryall RL (1995) Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in human undiluted human urine in vitro. Urol Res 23:53
5. Dussol B, Geider S, Lilova A, Léonetti F, Dupuy P, Daudon M, Berlan Y, Dagorn J, Verdier J (1995) Analysis of the soluble organic matrix of five morphologically different kidney stones. Evidence for a specific role of albumin in the constitution of the stone protein matrix. Urol Res 23:45
6. Finlayson B, Vermeulen CW, Stewart EJ (1961) Stone matrix and mucoprotein in urine. J Urol 86:355
7. Fraij BM (1989) Separation and identification of urinary proteins and stone-matrix proteins by mini-slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Clin Chem 35:658
8. Franzen A, Heinegard D (1985) Isolation and characterization of two sialoproteins present only in bone calcified matrix. Biochem J 232:715
9. Hess B (1991) The role of Tamm-Horsfall glycoprotein and nephrocalcin in calcium oxalate monohydrate crystallization processes. Scanning Micros 5:689
10. Hoyer JR (1994) Uropontin in urinary calcium stone formation. Miner Electrolyte Metab 20:385
11. Khan SR, Hackett RL (1987) Crystal-matrix relationships in experimentally induced urinary calcium oxalate monohydrate crystals, an ultrastructural study. Calcif Tissue Int 41:157
12. Khan SR, Hackett RL (1993) Role of organic matrix in urinary stone formation: an ultrastructural study of crystal matrix interface of calcium oxalate monohydrate stones. J Urol 150:239
13. Kohri K, Umekawa T, Kodama M, Katayama Y, Ishikawa Y, Takada M, Katoh Y, Kataoka K, Iguchi M, Kurita T (1990) Inhibition effect of glutamic acid and aspartic acid on calcium oxalate crystal formation. Eur Urol 17:173
14. Kohri K, Suzuki Y, Yoshida K, Yamamoto K, Amasaki N, Yamate T, Umekawa T, Iguchi M, Sinohara H, Kurita T (1992) Molecular cloning and sequencing of cDNA encoding urinary stone protein, which is identical to osteopontin. Biochem Biophys Res Commun 184:859
15. Leal J, Finlayson B (1977) Adsorption of naturally occurring polymers on calcium oxalate surfaces. Invest Urol 14:278
16. Lentner C (1982) Units of measurement, body fluids, composition of the body, nutrition. In: Ciba-Geigy, 8th edn., Vol 1, p 57
17. Lian JB, Prien EL, Glimcher JMJ, Gallop PM (1977) The presence of protein-bound γ-carboxyglutamic acid in calcium-containing renal calculi. J Clin Invest 59:1151
18. Mckee MD, Nanci A, Khan SR (1994) Ultrastructural immunodetection of osteopontin and osteocalcin as major matrix components of urinary calculi. J Bone Miner Res 9 [Suppl] 1: S379
19. Morrissey JH (1981) Silver stain for protein in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307
20. Morse RM, Resnick MI (1988) A new approach to the study of urinary macromolecules as a participant in calcium oxalate crystallization. J Urol 139:869
21. Morse RM, Resnick MI (1989) A study of the incorporation of urinary macromolecules onto crystals of different mineral compositions. J Urol 141:641

22. Shevock PN, Khan SR, Hackett R (1993) Urinary chemistry of the normal Sprague-Dawley rat. Urol Res 21:309
23. Shiraga H, Min W, Vandusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG, Hoyer JR (1992) Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 89:426
24. Stacholy J, Goldberg EP (1985) Microstructural matrix-crystal interactions in calcium oxalate monohydrate kidney stones. Scanning Electron Microsc II:781
25. Stapleton AMF, Ryall RM (1995) Blood coagulation proteins and urolithiasis are linked: crystal matrix protein is the F1 activation peptide of human prothrombin. Br J Urol 75:712
26. Stapleton AMF, Simpson RJ, Ryall RL (1993) Crystal matrix protein is related to human prothrombin. Biochem Biophys Res Comm 195:1199
27. Suzuki K, Moriyama M, Nakajima C, Kawamura K, Miyazawa K, Tsugawa R, Kikuchi N, Nagata K (1994) Isolation and partial characterization of crystal matrix protein as a potent inhibitor of calcium oxalate crystal aggregation: evidence of activation peptide of human prothrombin. Urol Res 22:45
28. Vermeulen CW, Lyon ES (1956) Mechanisms of genesis and growth of calculi. Am J Med 45:684
29. Warpehoski MA, Buscemi PJ, Osborn DC, Finlayson B, Goldberg EP (1981) Distribution of organic matrix in calcium oxalate calculi. Calcif Tissue Int 33:211
30. Worcester EM, Nakagawa Y, Wabner CL, Kumar S, Coe FL (1988) Crystal adsorption and growth slowing by nephrocalcin, albumin, and Tamm-Horsfall protein. Am J Physiol 255:1197
31. Worcester EM, Blumenthal SS, Beshensky AM, Lewand DL (1992) The calcium oxalate crystal growth inhibitor protein produced by mouse kidney cortical cells in culture is osteopontin. J Bone Miner Res 7:1029