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MODULATORS OF URINARY STONE FORMATION

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1. ABSTRACT

Urine contains compounds that modulate the nucleation, growth and aggregation of crystals as well as their attachment to renal epithelial cells. These compounds may function to protect the kidneys against: 1, the possibility of crystallization in tubular fluid and urine, which are generally metastable with respect to calcium salts, 2, crystal retention within the kidneys thereby preventing stone formation and 3, possibly against plaque formation at the nephron basement membrane. Since oxalate is the most common stone type, the effect of various modulators on calcium oxalate (CaOx) crystallization has been examined in greater details. Most of the inhibitory activity resides in macromolecules such as glycoproteins and glycosaminoglycans while nucleation promotion activity is most likely sustained by membrane lipids. Nephrocalcin, Tamm-Horsfall protein, osteopontin, urinary prothrombin fragment 1, and bikunin are the most studied inhibitory proteins while chondroitin sulfate (CS), heparan sulfate (HS) and hyaluronic acid (HA) are the best studied glycosaminoglycans.

Crystallization modulating macromolecules discussed here are also prominent in cell injury, inflammation and recovery. Renal epithelial cells on exposure to oxalate and CaOx crystals produce some of the inflammatory molecules such as monocyte chemoattractant protin-1 (MCP-1) with no apparent role in crystal formation. In addition, macrophages surround the CaOx crystals present in the renal interstitium. These observations indicate a close relationship between inflammation and nephrolithiasis.

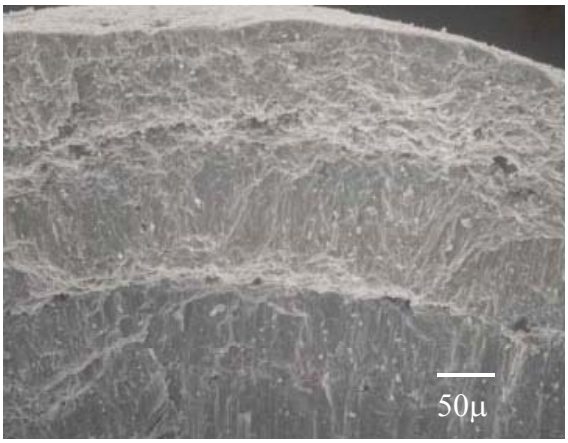

Figure 1. SEM of the fractured surface of calcium oxalate monohydrate stone. The stone is compact, shows distinct concentric laminations and radial striations. Concentric laminations are the result of outward growth of the stone. Radial straitions are the result of plate-like habit of calcium oxalate monohyydrate crystals.

2. INTRODUCTION

As a direct consequence of the renal function of water preservation, urine becomes supersaturated with slightly soluble salts like calcium oxalates and -phosphates (1). Except for special cases, the glomerular filtrate is not supersaturated. Supersaturation for calcium phosphate (CaP) is first achieved in the loop of Henle (2), while that for calcium oxalate (CaOx) occurs in the distal tubules. These sites move along the nephron because of changes in the concentrations of calcium, phosphate or oxalate in the nephronic fluid, or variation in the nephron function (2, 3). High concentrations present in the deep papillary nephron segments may permeate to the basement membranes and renal interstitium notwithstanding the low transmembrane permeability of e.g. the thin loop membranes. When supersaturation is high enough and lasts long enough or promoters are present, crystals nucleate in the urine and are either excreted as crystalluria (4), or deposit in renal tissue, appearing as so-called Randall's plaques (5). Both types of nuclei may increase in size by growth and/ or aggregation (6). In most people, crystals formed in the urine are discharged without any discomfort (4, 7-10). In hyperoxaluric animal models and hyperoxaluric patients crystal deposits remain in the urinary space, causing tubular epithelial damage and formation of large aggregates (11-13). In addition in hypercalciuric stone formers subepithelial deposits are found in the loop of Henle (14). Tubular retentates may evolve into stone nidi, a base for stone growth (5), as seen in renal biopsies from hyperoxaluric stone formers (14). Randall's plaques remain just that in most people who never form stones (15); but in some, they may grow through the interstitium towards the papillary surface and become a nidus for stone formation (14).

The fact that the widespread occurrence of crystalluria and Randall's plaques leads to stone formation in much fewer people and then usually only once or twice a lifetime suggests there are mechanisms that ensure crystals pass harmlessly and plaques stay as plaques. These mechanisms act at all levels of crystallization and stone formation: supersaturation, nucleation, crystal growth, aggregation, crystal structure and habit, crystal surface properties and crystal interactions with epithelial cells. There are even mechanisms to remove crystals deposited in the renal interstitium. In hyperoxaluric rats and patients, calcium oxalate crystals, which were retained in the tubules and had entered the renal interstitium, were attacked by macrophages that produce enzymes to dissolve the crystalline material and break down the organic crystal matrix. (11, 13, 16). The macrophages appear to be attracted by chemokines such as monocyte chemoattractant protein-1 (MCP-1) produced by renal epithelial cells (17) and by crystal associated compounds like osteopontin (18, 19).

Many urinary compounds have a protective role, usually involving an affinity for the crystals or its constituents. Lowering salt ion concentrations decreases urinary supersaturation. Citrate can bind calcium; magnesium binds oxalate and the total urine ionic strength effects the solubility of calcium salts. Thus a mainstay of therapy has been to reduce supersaturation by dilution (drinking fluids), decrease excretion of stone ions (e.g. restricted oxalate and/or calcium intake) or increase excretion of chelators (alkali therapy to increase citrate excretion). The common occurrence of urine supersaturation and crystalluria has prompted studies on modulators of the processes beyond supersaturation. This review presents current knowledge of crystallization modulators summarizing our understanding of the involvement of urinary macromolecules in various phases of crystallization in the urine as well as crystal retention, crystal removal and stone development. Special emphasis is given to the calcium salts CaOx and CaP, since they are the major crystals in most stones and, as such, have been investigated extensively.

3. THE STONE

Much can be learned from the stone structure. Stones can contain a variety of crystals including CaOx, CaP, uric acid, struvite, and cystine (6, 8, 9, 20). CaP crystals appear most frequently in both the urine and stones however, CaOx is the major crystal in most stones. Stones, particularly those containing CaOx or uric acid, have a compact structure (Figure 1). Their outer surfaces appear smooth at low magnification but reveal the presence of individual tabular or plate-like CaOx monohydrate (COM) crystals at higher magnifications (Figure 2). Crystal habits are generally not evident on surfaces exposed by cutting or fragmenting the stone (Figure 1). Such surfaces are typically stratified with radial striations and concentric laminations or layers, with radial striations being the predominant feature. Some of the striations run through many laminations while others are limited to only one. Many converge to a point at the base of a lamination mimicking the arrangement of petals in a flower. These points are suggested to be the nucleation sites of crystals.

Figure 2. Outer surface of calcium oxalate monohydrate stone. Edges of tabular crystals are clearly visible

The laminations are approximately 50-60 μm thick and in many stones can be easily separated from each other exposing the underlying surfaces. The latter show the same structure as the outer stone surface, with protruding tips of the tabular COM crystals frequently covered with amorphous to flaky matrix material. Overall it is a highly ordered structure. Many stones have a well-defined nucleus that is less ordered, with a granular and non-stratified appearance. It is generally occupied by spherulitic or amorphous CaP and/or aggregates of dumbbell shaped twinned COM crystals. CaP frequently fills the space between CaOx crystals as well as that in the concentric laminations.

Like other products of crystallization in biological systems (21), stones are a composite of crystals and organic material, often referred to as matrix (22-24). Matrix consists of macromolecules generally present in the urine. These molecules play a significant role in the development of kidney stones. Some of them promote crystal formation, growth, aggregation and retention, while others inhibit these processes. Their activity is often complex and depends on the urine conditions prevailing at the time of crystallization or retention. The same macromolecule can both promote and inhibit a process. For example macromolecules behave differently in solution than when they are attached or adsorbed to a surface. It may well be, that compounds free in solution cover a crystal surface and inhibit its growth or ability to aggregate while the same compound bound to a surface acts to accumulate salt ions and forms a template for the first nucleus. The latter will play a role when stone formation involves processes at cell surfaces and in the sub-epithelial space (3).

We first discuss the stone matrix emphasising crystal/ matrix associations in urinary crystals and stones. Then for low and high molecular weight (macromolecular) compounds present in urine and stone matrix we discuss their effects on crystallization and retention in the urinary tract. Although these compounds can both stimulate and inhibit crystallization and retention processes, they are usually referred to as inhibitors or modulators.

4. THE STONE MATRIX

The organic matrix of most urinary stones accounts for 2-3% of their total dry weight, rare matrix stones with 65% matrix contents notwithstanding. Boyce *et al.* defined and established the importance of stone matrix in urolithiasis, proposing that the matrix actively participates in the assembly of kidney stones (19). In their view, the matrix acts as a template and controls crystallization within its bounds. An opposite hypothesis was advanced by Vermeulen *et al.*, who viewed the matrix and its ubiquitous presence as merely coincidental, because stones form by crystallization in urine in the presence of large macromolecules (25, 26). According to them the matrix is adventitiously acquired, primarily by physical adsorption of urinary mucoproteins on crystal surfaces. A third possibility we propose is that the role of matrix compounds is different in the formation of the stone center and in the subsequent build-up of the stone. The first is a short-term event involving crystal formation and retention. The second is a long term event occurring after a stone nidus has been formed and retained. Both events do not necessarily take place at the same site.

Solution depletion (27) and examination of crystals incubated in protein solutions by transmission electron microscopy (28) tested the theory of physical adsorption of urine proteins on surfaces of CaOx crystals. Results showed proteins have a strong affinity for CaOx crystals. Adsorption of anionic proteins was sensitive to calcium ion concentration, whereas cationic protein adsorption depended upon the oxalate ion concentration with temperature and pH playing only a minor role. Proteins formed a discontinuous coat around the crystals ranging in thickness from 10 to 20nm. It has been suggested that newly formed crystals with a macromolecular coat are less likely to dissolve during the routine urinary ionic and pH changes and therein may lie the importance of matrix in stone formation (24).

4.1. Morphology

Stone matrix is extremely insoluble, which has hampered chemical identification of the constituent molecules. However, this also provided an opportunity for microscopic examination of the mostly EDTA-insoluble matrix and identification of components using a variety of stains and antibodies (29). Morphological examination of decalcified and intact stones shows that matrix is pervasive (Figure 3), distributed throughout the stones, and has amorphous and fibrous components (22-24). The crystal matrix association is so intimate that the dipyramidal habit of CaOx dihydrate (COD), the monoclinic or plate-like habit of COM and spherulitic habit of CaP stay intact after total removal of the mineral content (24). Scanning electron microscopic examination of decalcified COM stones showed the matrix to be organized in concentric layers of 2-5 μm thick. The thickness was uniform throughout the circumference and smaller than the laminations seen in an intact stone. Successive layers appeared as rings of an onion bulb with little or no space in between. They consisted of loosely or tightly matted fibers and contained empty columns representing crystal ghosts, presumably formed by

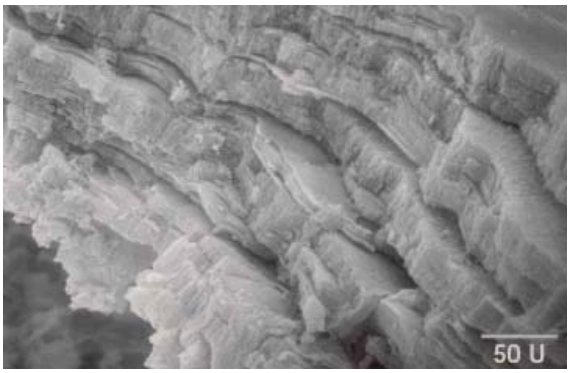

Figure 3. Fractured surface of demineralized calcium oxalate monohydrate stone. Demineralization was accomplished by treating the stone pieces with EDTA. Once stone became transluscent, it was dried and fractured to expose the interior. Microanalysis of the stones showed no calcium. A comparison with Figure 1 indicates the ubiquitous nature of the organic matrix. Increase in concentric lamination and loss of radial striations suggests that organic matrix became incorporated during the outward growth of the stones.

Table 1. Macromolecules detected in CaOx/CaP stones and/or crystals

Macromolecules	**Reference Number**
Proteins	
Albumin	41, 262
α-1-microglobulin	303
α-1-acid glycoprotein	265
α and γ globulins	41, 262
α-1-antitrypsin	304
Apolipoprotein A1	265
β-2-microglobulin	265
Calprotectin (Calgranulin)	32, 305
Haemoglobin	306
Inter-α-Inhibitor	42,194,195
Nephrocalcin	157-159
Neutrophil elastase	306
Osteopontin (Uropontin)	220
Porin	39
Renal lithostatin	265
Retinol binding protein	265
Superoxide dismutase	39
Tamm-Horsfall Protein	41
Transferin	258
Urinary prothrombin fragment-1	240-242
Glycosaminoglycans	
Heparan sulfate	35-38
Hyaluronic acid	
Lipids	
Phospholipids, Cholesterol, Glycolipids	30, 33, 284
Small molecules	
Pyrophosphate	307

dissolution of tabular COM crystals. Crystal ghosts were often arranged radially in relation to the stone centre, reminiscent of the radially arranged crystals in the intact stones.

Examination of the concentric layers by transmission electron microscopy confirmed that they contained radially arranged columnar crystal ghosts surrounded by an amorphous electron dense coat and embedded in a fibrous matrix. Electron dense material was also found inside crystal ghosts. Whether this points to an actual presence of organic material inside the original crystal or to a situation that the large original crystal was actually composed of smaller crystallites covered with organic material is still a point of debate. Cellular degradation products including degenerating nuclei, mitochondria, endoplasmic reticulum and membrane fragments as well as vesicles occupied the intercrystalline spaces (24). The layered matrix was sudanophilic (30) and stained positive with periodic acid Schiff (PAS) and colloidal iron (31), indicating the presence of lipids, glycosaminoglycans (GAG) and proteins. Examination of decalcified stones using antibodies against osteopontin (OPN) and calprotectin showed them to stain both the stone center and concentric laminations (32). Ultrastructural immunodetection showed OPN and osteocalcin to be major components of the matrix of human kidney stones as well as CaOx crystals and stones experimentally induced in male rats (16). OPN was detected both inside the crystals as well as on their surfaces. Ultrastructural examination of decalcified stones also showed the crystal associated matrix to stain positive with malachite green indicating the presence of phospholipids (33).

4.2. Chemical Composition

The organic matrix of urinary stones contains lipids, GAG's, carbohydrates and proteins. Proteins comprise approximately 64% of the matrix. Table 1 lists the compounds, which have been identified in matrices of urinary stones. Most of them are proteinaceous in nature. A number of other proteins have also been detected but not identified. Initially lipids were not recognized as constituent of stone matrix (22) even though detected as an osmiophilic substance during histochemical examination of decalcified stones. As discussed later in the review, recent studies have provided compelling evidence that lipids are an important component of stone matrix and play a significant role in stone formation.

All urine macromolecules can become part of stone matrix, but only some are there because they have participated in crystallization and stone formation. This appreciation led investigators to study crystallization *in vitro;* using freshly collected urine to determine the macromolecules that become a part of the crystal matrix (34).

4.2.1. Glycosaminoglycans

GAGs can account for up to 20% of the stone matrix (35). Heparan sulphate (HS) and hyaluronic acid (HA) are the two major GAGs in the matrix of both stones and CaOx crystals formed in urine (36, 37). The most abundant urine GAG, chondroitin sulphate (CS) was not found in these matrices, indicating selective incorporation (38). Keratan sulphate and dermatan sulphates are present in trace amounts.

Table 2. Stone Matrix Composition (% of EDTA soluble matrix) and Lipid Constituents (mg/g stone)

Stone Type	Protein	Lipid (% matrix)	Total Cholesterol	Cholesterol Ester	Triglycerides	Glycolipids	Phospho-lipid (AMF)	Phospho-lipid (VBR)
Struvite (n=5)	74%	26%	1.53±0.72	0.21±0.25	10.71±9.17	0.13±0.05	0.57±0.5	0.06±0.04
Calcium Oxalate (n=5)	20%	80%	0.64±0.27	0.37±0.10	1.64±0.6	0.16±0.06	0.18±0.1	0.05±0.03
Calcium Phosphate (n=3)	33%	67%	0.76±0.5	0.37±0.26	1.45±0.13	0.17±0.1	0.31±0.23	0.05±0.02
Uric Acid (n=5)	75%	25%	0.2±0.07	011±0.04	1.6±0.34	0.09±0.03	0.08±0.03	0.03±0.01

Table 3. Lipids Present in the Matrix of Crystals Produced in Normal Human Urine (mg/ total crystal/24 hours)

Crystal Type	Cholesterol	Cholesterol Ester	Triglycerides	Glycolipids	Phospholipids
Calcium Oxalate	0.37±0.09	0.20±0.04	3.11±2.9	1.15±1.20	0.09±0.04
Calcium Phosphate	0.27±0.10	0.21±0.03	2.51±2.4	1.35±1.04	0.02±0.01

4.2.2. Proteins

More than twenty individual proteins have been detected in the matrix of various types of stones. While most of them have been identified (Table 1), some still remain nameless (39) and a few await confirmation of their identity (40). Human serum albumin (HAS), α and γ-globulin and Tamm-Horsfall Protein (THP) were the first proteins identified in stone matrix (41). Albumin is a major component of the matrix of all stone types including CaOx, uric acid, struvite and cystine. It is also found in the matrix of CaOx and CaP crystals precipitated from human urine and it is more pronounced in crystals induced in stone formers urine (42, 43). Both CaOx and CaP crystals are known to adsorb HAS. THP is not always detected in stones and even then in only minor quantities, 0.002-1.04 mg/g (w/w) of stone (44). We found THP in the matrix of CaOx crystals and as a major component in the matrix of CaP crystals formed in human urine. We discovered that THP associated with CaOx crystals is easily removed by washing the crystals with sodium hydroxide solution (43, 45), indicating THP's loose interaction with the CaOx crystal surfaces. Ultrastructural investigations of human CaOx urinary stones (unpublished results) and CaOx nephroliths induced in an animal model supported the hypothesis that THP is not included in the crystals (46, 47). This may explain THP's scanty presence in the stone matrix.

Of the other proteins listed in Table 1, osteopontin (OPN), α-1- microglobulin, urinary prothrombin fragment-1 (UPFT-1), and light and heavy chains of inter-α-inhibitor have been identified in the matrix of CaOx and CaP crystals precipitated from the urine of normal and stone forming individuals (43). Ultrastructural examination reveals OPN to be pervasive in the crystals and stones and a key component of the matrix of CaOx stones (18, 48). More OPN is present in CaOx monohydrate stones (800 μg/100mg stone) than in COD stones (10 μg/100mg stone).

4.2.3. Lipids

Lipids are an integral part of the organic matrices of all mineralized tissues and pathological calcifications (49-51). Even though they account for a small proportion of the matrix; 7-14% in bone, 2-6% in dentin, 12-22% in newly mineralised enamel, 9.6% in submandibular salivary gland calculi and 10.2% in supragingival calculi (49-54), lipids play a significant role in calcification. They promote crystal nucleation, modulate growth and aggregation and become incorporated in growing calcifications.

The matrix of all stones examined to date, including struvite, uric acid, CaOx and CaP contains lipids (30, 33). The protein to lipid ratio is, however, higher in the matrix of struvite and uric acid stones than in CaOx and CaP stone matrix (Table 2). Even though there are no significant differences in types of lipid, the matrix of struvite stones contains more cholesterol, cholesterol ester and triglycerides than the other three stone types. One dimensional thin layer chromatography separated and identified various phospholipids and glycolipids including sphingomyelin (SM), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), cardiolipin (CL) and trace amounts of phosphatidylserine (PS) in all stone matrices. Occasionally, the stone matrix also contains phosphatidyl inositol (PI), lyso-PC, lyso-phosphatidic acid (PA) and lyso-PE. In all stones glycolipids include gangliosides, D-sphingnosine, and glucocerebrosides. In addition, the struvite stone matrix contains sulfatides and digalacto diglycerides while CaOx and CaP stone matrix contains cerebrosides 1 and 2 and digalacto-diglycerides. All stone matrices contain both complexed and non-complexed lipids. The amount of complexed lipids is highest in CaP and lowest in uric acid stones. Both complexed and non-complexed lipids contain cholesterol, triglycerides, phospholipids and gangliosides. Both CaOx and CaP crystals induced in the urine contain lipids (33). There are no significant differences in either the nature of lipid constituents or the amounts of lipid per gram of crystal between the two types of calcific crystals (Table 3). Glucocerebrosides are the most common glycolipids and SM the most common phospholipid. Gangliosides are the second most common glycolipid, PC and PE the most common phospholipids. Determinations of lipids in the urine before and after experimental induction of CaOx crystals show that the formation of crystals depletes the

urine of its phospholipids indicating its incorporation in the crystal matrix. Almost all the urinary phospholipids become incorporated during the formation of crystals (33).

5. MODULATORS OF CRYSTAL FORMATION AND RETENTION

In urine, three classes of modulators are recognized; low MW compounds like citrate and pyrophosphate; glyco-proteins; high MW non-protein compounds like acid mucopolysaccharides, glycosaminoglycans and various types of lipids. They modulate crystal formation and retention in the urinary tract either directly by interacting with the crystal or indirectly by influencing the urinary environment. From crystallization experiments with urine, it appears that in non-stone formers the concerted actions of these compounds ensure that: 1, the crystals formed remain unaggregated and small enough to be excreted (55, 56); 2, the crystals have a reduced affinity for epithelial cells (57-59) and; 3, the crystals if needed are easily recognized and removed by macrophages (60). Crystallization in confined spaces, e.g. simulating Randall's plaque formation at the basal membrane below the tubular epithelium has been less studied but even here, inhibitors can decrease crystal growth rates (61, 62). Which inhibitors are the most effective? The first approach to answering this question has been to identify individual urine compounds and test their "inhibitory" potency in crystallization and cell-culture systems. The next problem has been to translate these data to the whole urine situation where singular inhibitors may also co-operate or compete with each other and where restrictions posed by the kidney and urinary tract itself (flow-rates, residence time and changing urine composition) affect their inhibitory and stimulatory powers. We will discuss the actions of individual modulators in inorganic solutions as well as in the urinary conditions.

Undiluted whole urine strongly affects calcium salt nucleation, crystal growth and crystal aggregation. When preformed CaOx crystals were added to supersaturated whole-undiluted urine their growth was almost completely stopped. Crystal growth only occurred when the supersaturation was drastically increased by adding extra oxalate (2). Urine has an overabundance of inhibitors. Tested in vitro as single compounds, some are clearly more effective than the others, however experimental data suggest that when the most efficient compounds are lacking, others readily take over. For instance, the low MW compound citrate can inhibit crystal growth very effectively at concentrations between 0.1 and 1 mM. When citrate was added at these concentrations to urine, however, it did not change the growth inhibitory action of that urine (55). In studies of large groups of stone formers and healthy controls where urine was tested in a 1:5 dilution, approximating the degree of dilution existing in the collecting ducts, both urine from stone formers and normal subjects strongly inhibited CaOx crystal growth (55, 56, 63). When all macromolecules were removed by ultrafiltration, the degree of crystal growth inhibition was only slightly reduced (64).

In vitro tests have confirmed that macromolecules are the most effective inhibitors of crystal growth. Apparently the low MW compounds take over the inhibitory function when the high MW compounds are gone. An additional effect of crystal growth inhibition may be that supersaturation will persist longer and the process of nucleation will have more time to proceed (65). How relevant this is, in view of the short transit times of urine through the nephron (a few minutes) (12), is not clear.

Normal urine can also strongly inhibit crystal aggregation. This function is reduced in single stone former urine and severely reduced in recurrent stone former urine (55, 56). Aggregation is important as it can lead to particle retention, just like crystal cell interactions and disturbed flow conditions (12). The inhibition of aggregation in urine is correlated to the citrate concentration (56). However, in ultrafiltered urine this relationship is gone (63). Apparently citrate modulates the effect that high MW compounds have on crystal aggregation. In addition it was found that the urinary macromolecular fraction (>10.000 D MW) of single stone formers inhibited crystal aggregation less than that of normals and even less by those from recurrent stone formers (65). In this study 70-90% of the inhibitory activity was destroyed by proteinase treatment. Citrate has been shown to improve the inhibitory effect of THP on crystal aggregation (66).

Overall it appears that urine contains numerous components, both small and large that compete and co-operate in modulating crystallization and inhibiting stone formation.

5.1. Low Molecular Weight Compounds

5.1.1. Pyrophosphate and Bisphosphonate

Pyrophosphate is present in urine at concentrations of 15-100μM (Table 4). In a seeded crystal growth system (Table 5), it inhibits COM crystal growth by 50% at 16-20 μM (67-70). It can also inhibit COM crystal growth inside a gel matrix (71) and effectively inhibits the growth of CaPs (72, 73). If it is equally efficient in urine it can contribute 50% crystal COM growth inhibition in the collecting ducts (5 times dilution) and up to 80% in the urine. This efficacy prompted interest in therapies that raise the urine output of pyrophosphate and in non-biodegradable pyrophosphate analogues, bisphosphonates. These inhibit COM crystal growth at least as good as pyrophosphate, 50% inhibition at 1-20 μM concentrations (74). Another feature of pyrophosphate, 2.0 10^{-4} mol/l, which it shares with citrate, 1.0 10^{-3} mol/l, is that COD is preferentially formed in its presence. The critical pyrophosphate concentration above which COD formation is prevented may be lowered to the physiological range by adding citrate (75). This is of interest as COD is the major crystal phase in normal crystalluria (76) while there is more COM in stoneformer crystalluria and COM predominates in stones.

The effects of pyrophosphate and bisphosphonates on crystal aggregation are more complex. Pyrophosphate increasingly inhibits crystal aggregation at increasing concentrations (68). At its concentration range in the collecting ducts it could contribute to the whole urine effect on aggregation. While some bisphosphonates

Table 4. Characteristics of low MW compounds and GAGS [Reference numbers 56,67,82-86,110,125,127,130, 132, 307-317]

Compound	MW	Urinary excretion, mg/24h avg (range)	
		Control	Stone Fomer
Total GAGs, mg/24h		23-28 (0-50)	23-(0-53)
ChondroitinsulfateA, C[1]	5-20 kDa	14 (8-18)	14 (4-19)
ChondroitinsulfateC[1]			
Keratansulfate[1]	5 kDa	2 (0.5-7)	2 (0.5-7)
Dermatansulfate (Chs-B)[1]		1 (0.5-2)	1 (0.5-2)
Heparansulfate[1]		5 (2-14)	5 (3-14)
Hyaluronic acid[1]	up to 10^6 Da[3]	3 (1.8-7.5)	3 (1-8)
Pentosanpolysulfate[2]	4-7 kDa		1-16
Pyrophosphate (µmoles)	178 Da	30-70 (15-100)	36-50 (8-94)
Citrate (mmoles)	192	3.8 (1.8-8.0)	2.4 (0.1-4.5)

[1] based on measurements of separate GAGs and on reported % of the total GAGs, [2] excretion rate at a 400 mg/ 24hr oral dose, 12% >100kDa, 64% 10-100, 24%<10kDa

Table 5. Modulator activity of pure compounds [Reference numbers 57,59,68-70,76, 106, 118,119,233,253,278-280, 313]

Concentration (M) for:	50%G.I.[1]	%G.I. in urine[2]	AggI [3]	Crystal-cell binding[5]	
				MDCK, mg/L	BSC-1, µM
Bisphosphonates	1-50 10^{-6}		Variable		
Chondroitin Sulfate A[1]	40-50 10^{-6}	15-20	no to small effect	>100	0.6
Chondroitin Sulfate C[1]	10^{-6}			>100	no effect
Citrate	$0.20.10^{-3}$	70	Strong inhibition		$0.2.10^{-3}$
Dermatan Sulfate(Chs-B)					0.1
Heparin	3-20 10^{-9}		no effect	0.5;3.1	0.002-0.015
Heparan sulfate[1]	6 10^{-9}	30-40	Strong inhibition	>100	0.1
Hyaluronic acid[1]	10^{-6}		no to small effect	>100	0.02
Keratan Sulfate[1]	10^{-6}				-
Pentosan Polysulfate&	2-6 10^{-6}	35[4]	no effect	2	0.02
Pyrophosphate	2-20 10^{-6}	50-60	Strong inhibition		

[1] as % reduction of growth rate in inhibitor-free control experiment, [2] the % growth inhibition(G.I.) in an in-vitro experiment by the average concentration present in urine, [3] Aggregation inhibition (AggI) techniques employed in literature are too diverse to allow for quantitation, [4] Expected concentration at 400 mg/day dose is 16 mg/l. Addition to urine at 10 mg/l increased % GI from 45 to 59% [127], [5] The dose needed for 50% reduction in the binding of COM crystals to quiescent layers of renal epithelial cell cultures of MDCk [57] and BSC-1 [59].

have a comparable effect, others show no effect, a stimulatory effect on aggregation or even a biphasic effect, inhibiting aggregation at low concentrations and stimulating it at higher concentrations (74). From experiments with a series of bisphosphonates where slight variations were made in their structure it was concluded that bisphosphonates bind to the crystal surface by a combined action of the two phosphonate groups and side chains in close proximity. Increasing the affinity for calcium of these side groups increased the capacity to inhibit crystal growth. The presence of two calcium binding phosphonate groups makes bisphosphonates to likely form large polynuclear complexes with calcium ions acting as a bridge (78). These complexes act as one macromolecular structure and inhibition of aggregation is reversed to stimulation (74). The complexes bind to more than one crystal at the same time and act as a bridge (viscous binding). The poor results with etidronate therapy for stone prevention and the severe renal side effects (79) may have been related to this feature of large complex formation. Viscous binding can also explain why some macromolecules may at the same time strongly inhibit crystal growth and strongly stimulate crystal aggregation (80). Bisphosphonates with a large side chain (steric hindrance) do not form such large complexes and do not show stimulation of crystal aggregation.

Growth inhibition by bisphosphonates also depends on their protonation state, thus on the pH and its pKa-values. The triply deprotonated form, present when the pH surpasses the pKa3-value, is the most effective in inhibiting crystal growth. A pH-dependency is also found for pyrophosphate and citrate. In the urine pH range ionic species of pyrophosphate are PP^{4-}, HPP^{3-} and $H2PP^{2-}$. The first two adsorb onto COM crystals (81) and will predominate at higher pH values. Variation of the pKa3 value of a bisposphonate increases its activity at low urine pH values and might reduce its anti bone resorptive capacity. It may be possible to construct a bisphosphonate that strongly inhibits CaOx crystal growth and crystal aggregation at the

Table 6. Modulating effects of whole urine tested in 1:5 dilution [56,78,315]

Urine	%G.I.	AggI, minutes	Solubility, mM	Cell-adhesion
Normal	85±2	275±23	0.237±0.005	
SF, all	78±2	172±13	0.236±0.012	
1 stone	79±2	205±18	0.2350±0.014	
2-5 stones	77±3	160±16	0.240±0.015	
>5 stones	76±2	115±12	0.220±0.014	
Ultrafiltered SF[1]	76±3	80±8	0.235±0.013	
UMMS, adults[2]				67
UMMS, children[2]				49

1 all molecules with MW >30kDa removed, 2 molecules of > 3 kDa MW added to conluent MDCK cell-layer in a final concentration of 100 mg/l

urine pH levels and does not interfere with bone resorption activity at the low pH levels existing under active osteoclasts. Development and application of such compounds are future projects.

Since pyrophosphate is an effective inhibitor under non-urine conditions, several groups have investigated if stoneformers have a low urine pyrophosphate excretion. Pyrophosphate enters the urine in the glomerular filtrate. The plasma concentration is 2-3 µM, of which 70-80% is ultrafiltrable. The urine excretion rate is variable. In male non-stone formers the concentration averages 20-40 µM, the 24hr excretion rate is 30-60 µmoles (range 15-98 µmoles) (Table 4). Some investigators found that the 24hr pyrophosphate excretion was unchanged in stone formers, 36 µmoles/ 24hr (range 8-94), although they noted some variation with types of hypercalciuria (67). Other groups reported a decreased average excretion (51 in stone formers versus 71 in controls) (82), a decreased average pyrophosphate/ creatinin ratio in stone formers (83) or a decreased pyrophosphate excretion as single abnormality in 12% of the stone formers (84). Invariably, however, the range of excretion rates was comparable to that of controls. Women tended to have a higher average pyrophosphate excretion, but this was not significant (4.23 ± 3.34 versus 1.98 ± 1.0). In female stone formers and male stone formers the ppi/creat ratio was comparable, 2.16 ± 2.27. (84). Overall the data does not conclusively show a lower pyrophosphate excretion in stone-formers.

It is nevertheless possible that increasing pyrophosphate excretion raises the growth inhibitory power of urine and as such is beneficial. Two problems then occur, can the excretion of pyrophosphate in urine be manipulated and does it contribute to the inhibitory power of whole urine? Pyrophosphate excretion can be increased by oral orthophosphate therapy (85) but this therapy has not proven effective in preventing stone formation. Treatment with neutral potassium phosphate (Urophos-K)(86) and with diclofenac sodium (82) (a non-steroidal anti-inflammatory drug) also increased pyrophosphate excretion. The first also increased citrate excretion and the ability of urine to inhibit crystal aggregation and reduced the propensity for spontaneous nucleation of brushite (86). It has not been shown that it increases the inhibition of crystal growth in urine. Part of the efficacy of UrophosK in preventing urolithiasis may be related to these features.

5.1.2. Citrate

Citrate inhibits COM crystal growth at concentrations above 0.1 mM (66), which is in the range of its concentration in the loop of Henle (2). It is also inhibits crystal growth in a gel matrix (71). Citrate may contribute to crystal growth inhibition at sites where other, macromolecular, modulators have not entered the fluid yet.

Citrate also affects crystal aggregation, both in solution (68) and in a matrix situation (77). Tested as single modulator present, citrate inhibits crystal aggregation at concentrations above 0.1mM (68), and thus could be active up to the loop of Henle. However, this data cannot be directly extrapolated to the whole urine situation. When whole urine is tested in a 1: 5 dilution (the Dilution State in the collecting duct) urine is found to strongly inhibit crystal aggregation (Table 6), and there is a strong correlation with the urine citrate concentration (55, 56). But when all macromolecules are removed and citrate remains, the urine loses most of its capacity to inhibit crystal aggregation and the relationship between crystal aggregation inhibition and citrate concentration is lost (63). The conclusion is that citrate acts on crystal aggregation by potentiating macromolecules in urine.

An increase in citrate excretion may be relevant by itself. A decreased urine excretion of citrate, hypocitraturia, is found in a majority of stone formers. Correction of citrate excretion by alkali therapy in hypocitraturic recurrent stone formers resulted in correction of their decreased ability to inhibit crystal aggregation (55, 56, 63). In a meta-analysis of published data, alkali therapy was the only therapy which gave a significant decrease in stone formation rate (87). Alkali therapy also increases the urine pH. This in itself could be beneficial as inhibitors like citrate and pyrophosphate are more effective at pH values around 7.

Finally, it is not known if citrate, pyrophosphate and bisphosphonates *in-vivo* can affect crystal-cell interactions. *In-vitro*, citrate at a concentration of 0.2 mM decreased the binding of COM crystals to cultures of the renal epithelial cell line BSC-1 by 50% (59). The normal concentration of citrate at the site where crystals are likely to be present and interact with epithelial cells, the distal duct and collecting ducts, is up to 0.9 mM. Citrate might thus help prevent crystal-cell interaction *in-vivo*. Hypocitraturia could thus cause particle retention in the

Table 7. Distribution of individual GAGs as % of total GAGs population [35-37]

Compound	Urine		Cystals	Stone
	control	SF	Semi-quantitive	Semi-quantitive
Chondroitin sulfate	30-68	14-70	0[1]	0
Heparan sulfate	8-51	9-50	+++	+++
Hyaluronic acid	3-23	5-32		++
Keratan sulfate	2-27	2-21		+
Dermatansulfate	1-8	1-6		

[1] When Heparan Sulfate is absent from urine, Chondroitin sulfate can become included into crystal matrix

nephron both by increased formation of large aggregates and increased crystal-cell interactions, especially when it coincides with conditions that cause damage to these epithelial cells and render them susceptible for crystal binding (88).

5.2. High Mw Compounds

5.2.1. Glycosaminoglycans (GAGS)

In 1684 Anton von Heyde discovered the presence of a mucoprotein matrix in stone (89). Later, urine was found to contain many different anionic proteins and non-protein anions like (GAGS), RNA and acid mucopolysaccharides. Most prominent are the GAGS, polyanionic compounds with varying MW of usually18-40 kDa but up to 10^6 Da. GAGS can enter the urine by filtration, by release from the glomerular basement membrane (90), from the surface of the tubular epithelial lining and the urothelium further down the urinary tract, including the bladder (91). Well-known GAGS include heparin (not present in urine) and the urinary GAGS heparan sulfate (HS), chondroitin sulfate A B and C (CS-A, CS-B, CS-C) dermatan sulfate (DS), keratan sulfate (KS) and the non-sulfated hyaluronic acid (HA) (Table 7). Some, but not all urinary GAGS are found in crystals and stones (34-38). Is this selective inclusion due to differences in excretion rate and/or the affinity for calcium salt crystals?

Exact determination of the excretion rate of polyanions has been a problem. Total GAGs excretion can be measured with several methods (92). Alcian blue and cetylpyridinium chloride precipitate all polyanionic macromolecules including GAGS, THP, acid mucopolysaccharides plus anionic glyco-proteins and RNA. In early studies recurrent stone formers were found to excrete less of this mixture (92). When total hexuronic acid content of the precipitate is measured (93) an indication of the total amount of free plus protein bound GAGs is obtained, expressed as µmoles of glucuronic acid. (DS is missed because it contains iduronat instead of glucuronate.) Such studies show that in non-stone formers total GAGs excretion varies between 0 and 50 µmoles glucuronic acid/ 24hr. Some studies show a decreased excretion of GAGs in stone formers (95-105), however; at least an equal number of studies find comparable GAGs excretion by stone formers and non-stone formers (79, 106-115). The differences in results may relate to differences in employed techniques, patient selection, epithelial damage or the contribution of bladder excretions to the total GAG pool (93). The finding that excretion of uronic acid containing compounds (mainly GAGS) increases with age (from 0 to 15 years) (94, 116) may well be related to the increase of bladder surface area with age. One study showed no change for the total stone former group but decreased GAGs excretion in recurrent stone formers (83). Overall there is no conclusive evidence that differences in total GAGs excretion exist or play a role in stone formation. In view of the bladder origin of some urinary GAGs, it is not possible to estimate the concentration of GAGs in tubular urine.

Another approach has been to look at individual GAGS, by a combination of gel electrophoresis, enzymes that cleave specific GAGS and antibodies for specific GAGs or binding proteins (e.g. HA binding protein). Overall these studies do not show consistent changes in GAGs patterns in stone patients (92). The numbers given in Table 4 are combined data from the literature.

5.2.1.1. Inhibitory Actions

Although quantity does not appear to play a role, some data indicate that the quality of GAGS may vary. Urinary macromolecules and urine from children inhibit crystal aggregation better than urine of adults. The macromolecule fraction of pediatric urine contained more GAGs (116). GAGs from stone formers had an increased nucleation promoting activity but similar crystal growth inhibitory activity (83). The first appeared related to a changed action of HA in stone formers (118). However CS of healthy individuals also showed a basal crystallization-promoting property (119). To put such data in perspective you must first know how the several GAG species affect crystallization in inorganic solutions and under urine conditions. Table 5 summarizes data from the literature on the effect of GAGs on crystal growth, aggregation and nucleation when tested in inorganic solutions. Under these conditions the non-urine GAG heparin is the most effective on a molar basis. Of the GAGs present in urine HS is most effective followed at a distance by CS and HA. The heparin analog pentosan polysulfate has an efficacy between heparin and CS. As was shown for pentosan polysulfate, the inhibition of crystal growth does not change when pH is varied from 5 to 7 (120). With respect to crystal-cell interactions, coating of crystals by GAGs decreased the binding of crystals to renal epithelial cells in culture, but did not completely abolish it (58-60).

In view of the urine concentration of those compounds, HS should contribute the most to the crystallization inhibitory power of urine. CS should have some effect, as it is the GAG with the highest concentration. If the GAGs would act synergistically their contribution to the overall inhibitory power of urine should be significant. However, how do GAGs perform in urine?

5.2.1.2. Effects on Crystallization in Urine

The effect of 1% urine on crystal growth and aggregation was only slightly related to the uronic acid

content and overall the contribution of GAGs (65, 108, 115). Apparently the effects of GAGs in an inorganic solution cannot predict their actions in the urine situation. Overall it appears that CS is not active as an inhibitor in urine. In fact, it might even stimulate nucleation and stone-formation. HS has some effect in urine on crystal growth and aggregation and in addition may also promote nucleation. The synthetic GAG pentosan polysulfate also can inhibit crystal growth under urine conditions and reduces renal crystal deposition in rat models for stone formation. These data agree with the change in relative distribution of GAGs going from urine to crystals and stones (Table 7). While in urine CS is by far the most abundant GAG, crystals produced from whole urine ordinarily contain HS, none to some HA and no CS (35-37). Crystals produced in the absence of HS do contain CS, thus the changed distribution is a competition effect. In stone matrix 8-20% of total dry weight are GAGs. These stone GAGs include no CS, a large amount of HS and some HA (38, 39). Overall it appears that GAGs can have an "inhibitory" action in urine, but their contribution to the actual activity in urine seems small at best. Nevertheless, increasing the urine excretion of GAGs might add to the inhibitory power of urine. Another reason to try to increase GAG excretion may lie in their ability to influence Band-3 protein governed oxalate exchange (121). The oxalate self exchange is decreased in stone patient red blood cells and this can be corrected *in vitro* by adding HS. When patients received short-term oral GAGS treatment, the RBC oxalate self exchange is also normalized (122).

Furthermore, GAGs may interfere with crystal cell interactions. In cell-culture studies, preformed CaOx crystals do not bind to well developed MDCK cells (Distal tubule/ collecting duct origin). However the same cells during migration and proliferation do bind crystals, where HA appears to act as a crystal binding molecule (85). Adhesion of COM crystals to MDCK and BSC-1 cells was reduced by heparin, CS-A or B, HS, and HA, the non-sulfated polyglutamic acid and polyaspartic acid, nephrocalcin, uropontin, pentosan polysulfate and citrate but not CS-C and Tamm-Horsfall glycoprotein (THP) (57-59). Of the GAGs heparin and pentosan polysulfate were most effective at the lowest concentrations (Table 5). Also, coating of stents with heparin prevents encrustation of the stents when placed in a bladder for up to 120 days (123). Thus, under non-urine conditions GAGs appear capable of preventing crystal adhesion to cells and other surfaces. When crystals are pre-incubated in whole urine and then added to cell cultures in an inorganic solution, this reduced their binding to immobilised HA, confirming the crystal binding action of the latter. However, it did not significantly reduce the crystal-cell attachment (58). Just as was the case for crystallization inhibition, effects on crystal-cell attachment differ from inorganic solutions to semi-urine conditions. These combined data raise the question: can long term GAGs therapy decrease stone formation by decreasing the propensity for crystallization and crystal attachment to renal cells?

5.2.1.3. Therapeutic Uses

During GAGs therapy oxalate excretion decreased on a short-term basis (122). The long-term effect on oxalate excretion has not been studied. The only long-term study with administration of a GAG to stone formers was with pentosan polysulfate (PPS, Elmiron). In this study the stone formation rate in stone formers receiving 400 mg Elmiron daily was followed, first in an open study, later compared to a control group receiving standard advice. From studies with radioactive labelled Elmiron, it is known that only 8% of an intravenous 40 mg pentosan polysulfate dose reaches the urine. After an oral dose of 400 mg/day, plasma levels reached 0.02 to 0.05 microgram/ ml. The urinary excretion in a 24 hr period was 0.05 to 0.1% (1-2 mg). In rats 4% of a dose of 10 mg/kg/day of PPS reached the urine (124) whereas in man 1-4% reaches the urine (125). Thus of the 400 mg/day dose given in the clinical trial 4-16 mg may reach the daily urine. All of this will not be intact. In comparison, 50% of heparin is desulfated in the liver (126). If the same occurs with Elmiron, 2-8 mg of the daily dose of 400 mg will reach the urine. When we assume a MW of 5000 Dalton and a daily urine volume of 1.5 litres this means a concentration of 0.4 - 1.6 μM. Under non-urine conditions this is approximately the concentration that gives 50% inhibition of crystals growth. In the first report from 1986, 100 patients were started on 400 mg Elmiron/ day. A report was given on 70 patients that received Elmiron for a period of at least 12 months (127).

Long-term trials with Elmiron given for other indications show some side effects at doses of 150 to 450 mg/day (128). In this interim report six patients stopped because of gastrointestinal side effects and a trend was suggested that stone formation was reduced. In a second report from 1988, 100 patients were treated for a period of 12 to 56 months, with sixteen patients withdrawing. After this period 85% remained stone free. The results were however, obtained without a proper control group and thus contaminated with the stone clinic effect. The results encouraged an open study in which 121 patients were followed for 3 years. Data collected showed that 48% of the patients remained stone free, while 52 % continued to form stones. The stone formation rate was not statistically different from that in patients without treatment (129). The conclusion seems to be that although pentosan polysulfate shows potential when tested in the laboratory, when used in therapy at the dose of 400 mg/day it does not prevent stone formation. Possibly a higher dose is required but this may have the risk of considerable dropout due to side effects.

GAGs affect the morphology of COM crystals differently depending on the species. Chondroitin-6-sulfate produces elongated less wide crystals. Dermatan sulfate and heparin are incorporated into the crystals, while ChS-C is not. From experiments using dicarboxylates, a simple model of GAG molecules, showed that a distance between the side groups was important for their morphological effects (130).

In male rats a vitamin A-deficient diet caused a decrease in the concentration of urinary glycosaminoglycans and lesions of the cuboidal epithelium that covers the papillae (131). The plasma vitamin A levels in urolithiasic humans did not significantly differ from those in a control group. Nevertheless a significant increase in vitamin E and in the vit E/vit A ratio was

Table 8. Some Urinary Proteins With Potential to Modulate Crystal Formation and Retention

Protein	MW (kDa)	Renal Expression		Unique Features	Role in Crystallization	Urinary Excretion
		Normal	Hyperoxaluria			
Tamm-Horsfall Protein	80-100	TAL +	TAL ++	12% ASP	Promotor; Inhibitor of Aggregation	20-200 mg/day
Nephrocalcin	14	PT, TAL		2-3 Gla Residues	Inhibitor of Nucleation, Growth, Aggregation	5-16mg/l
Osteopontin	42-80	TDL, TAL, PS +	PT, TDL, CD, TAL, PS ++++	RGD Sequence	Inhibitor of Nucleation, Growth, Aggregation; Promoter of Crystal Adhesion	2.4-3.7mg/l
A-1 Microglobulin	31		PT, CD ++			5.34 mg/l
Calprotectin	36.5			Calcium Binding Domain		<50 µ g/l
Human Serum Albumin	68			Crystal Binding. Facilitates binding of other proteins.	Promotes nucleation	1.6-34.2mg/day
Urinary Prothrombin Fragment 1	31	TAL		10 GLA Residues	Inhibitor of Aggregation	13.4nM/day
Inter-α-Inhibitor		PT, CD +	PT, CD ++		Inhibitor of Nucleation, Growth, Aggregation and Crystal Adhesion	2-10mg/day
H1 (Heavy Chain1)	78			Sulfated GAGs		Yes
H2 (Heavy Chain2)	85					Yes
HI-30 (Bikunin)	30-35					Yes

Renal Expression (1+ - 4+) Based On Studies In Rats; Urinary Excretion In Normal Humans; Role In Crystallization Based Mostly On In Vitro CaOx Crystal Studies; ASP, Aspartic acid; GLA, γ- Carboxyglutamic acid; RGD, Arginine- Glycine-Aspartic Acid; PT, Proximal Tubule; TAL, Thin Ascending Limb Of The Loop of Henle; TDL; Thick Descending Limb Of The Loop of Henle; CD, Collecting Duct, PS, Papillary Surface

observed. These results could be related to a possible deficit of vitamin A in kidneys of stone formers, this being one of the diverse factors that can contribute to urolith development. Moreover, the deficit of important urinary crystallization inhibitors normally found in stone-formers, such as pyrophosphate and phytate, can also be related to the presence of low levels of renal vitamin A, which prevents the enzymatic degradation of such inhibitors (132).

5.2.2. Proteins

Table 8 lists major urinary proteins with known potential to influence crystallization of CaOx and/or CaP. Renal epithelial cells normally produce many of these while others are currently considered as plasma proteins. However, recent animal model and tissue culture studies demonstrate that the renal epithelial cells in the presence of hyperoxaluria and CaOx crystals also produce many of the so-called plasma proteins.

5.2.2.1. Tamm-Horsfall Protein

A number of excellent reviews have been written on Tamm-Horsfall protein (THP) involvement in nephrolithiasis (133-135). THP is one of the most abundant proteins in normal human urine and the major constituent of urinary casts. It was first isolated from the urine by Tamm and Horsfall and characterised as a glycoprotein that inhibits viral hemagglutination (136). Muchmore and Decker isolated a protein called uromodulin from the urine of pregnant women (137). Based on amino acid and carbohydrate analysis THP and uromodulin were shown to be identical (138). There is a considerable variation in daily urinary excretion of THP by both humans and rats. In humans it ranges between 20 to 100 mg/day with a daily urinary volume of 1.5 litres and in rats it ranges between 552 to 2, 865 µg/day with a daily urinary volume of 16.5 ml (139). When converted to mg/l rats excrete 34.5 + 38.6 to 180 + 38.6 mg/l THP in their urine. THP has a molecular weight of approximately 80 kDa with a tendency to aggregate to the polymeric form. Polymerisation is increased in the presence of free calcium ions, at high ionic strength and osmolality, and at low pH. Sialylated, sulfated and GalNac containing carbohydrates make up 30% of its weight. It contains 616 amino acid residues including approximately 50 half-cysteine molecules, which can be involved in disulfide bridge formation.

THP has been the subject of extensive research for its implication in stone formation. However, its exact contribution to urolithiasis remains unclear and the results of various studies have been controversial (133). Some studies indicated that THP promoted CaOx and CaP crystallization (140, 141). Other studies demonstrated that the macromolecule does not support CaOx crystallization and has no effect on spontaneous precipitation (142). Still other studies indicated that THP has no effect on nucleation or growth, but

Table 9. Expression of Crystallization Modulators: Results of Tissue Culture and Animal Studies using immunohistochemistry, PCR, in situ hybridization and microarray analysis

Macromolecules	Upregulated (Reference)	Downregulated (Reference)	No Effect (Reference)
Osteopontin	+ (224, 228, 237)		
Tamm-Horsfall Protein	+ (151)	+ (153)	+ (154)
CD 44	+		
Hyaluronic Acid	+		
Bikunin	+ (153, 180-183)		
Urinary Prothrombin Fragment-1	+ (249)		
Heparan Sulfate	+ (318)		

is a potent inhibitor of CaOx crystal aggregation (143-145). Hess *et al*. found that the addition of citrate reduced CaOx crystal aggregation by reducing the self-aggregation of THP isolated from stone formers urine (144). It is important to point out that low citrate or hypocitraturia is common in stone formers and can contribute to crystal aggregation and stone formation in this fashion. THP activity is controlled by its concentration, urinary osmolality and physicochemical environment of the urine (146). For example, at low concentrations, THP has a a minor effect on CaOx crystallization yet promotes it at higher concentrations. Also, when ionic strength was increased or the pH lowered the inhibition of CaOx monohydrate crystal aggregation by THP was decreased (143). Apparently, at high ionic strength, high THP concentration and low pH, the viscosity of THP increases due to its polymerisation. Several studies have shown that there is no significant difference in the daily urinary excretion of THP between normal subjects and CaOx stone formers (147). This fact led Hess *et al*. to hypothesize that THP of stone formers is structurally different from that of the healthy subjects (143). They showed that THP isolated from the urine of stone formers contained less carbohydrate (mainly sialic acid) than the THP obtained from control subjects (148). It has been suggested that the abnormality may be inherited, but sufficient evidence to support this concept is not available at this time. Studies have also shown differences in sialic acid contents and surface charge between THP from stone formers and normal individuals. Isoelectric focussing (IEF) studies have shown that THP from healthy individuals has a pI value of approximately 3.5, while THP from recurrent stone formers has pI values between 4.5 and 6 and the two exhibit completely different IEF patterns (149).

THP is exclusively produced in the kidneys. Based primarily on studies in rat kidneys, it is agreed that THP is specifically localized in epithelial cells of the thick ascending limbs of the loops of Henle (135, 150) and is generally not seen in the papillary tubules. When CaOx crystal deposits, the nephroliths, are experimentally induced in rat kidneys, THP is seen in close association with the crystals, both in the renal cortex as well as papillae (46, 47). However, THP is not seen occluded inside the crystals nor produced by cells other than those lining the limbs of the Henle's loop (151). There are no significant biochemical differences in the THP between one secreted by normal rats or rats with CaOx nephroliths. They have similar amino acid composition, carbohydrate contents, molecular weights and rates of urinary excretion. However, THP from nephrolithic rats has slightly less sialic acid contents, 20% of the total carbohydrate in nephrolithic rats vs. 26% in normal rats. In an aggregation assay, both the normal rat THP and nephrolithic rat THP reduced CaOx crystal aggregation *in vitro* by approximately 47%. Results of these rat model studies led to the conclusions that THP is most likely involved in controlling aggregation and that the major difference between normal and stone formers THP may be their sialic acid contents. However animal studies can not rule out THP's role in modulating crystal nucleation or growth. Another rat model study has shown increased expression of THP in kidneys following unilateral ureteric ligation, which caused tubular dilatation (152). The results indicated that THP expression in kidneys may be increased without crystal deposition and that increased expression in nephrolithic kidneys may be a result of crystal associated injury to the renal epithelial cells. Even rat model studies have provided controversial results for THP. One study shows decreased renal expression of THP during CaOx crystal deposition (153) while results of another study show upregulation of the THP gene (154).

5.2.2.2. Nephrocalcin

Nephrocalcin (NC) has been the subject of many reviews (155, 156). NC is a glycoprotein with a monomeric molecular weight of approximately 14 kDa and has a tendency to self-aggregate into a larger macromolecule and thus can exist as a dimer, trimer or tetramer with molecular weights of 23-30, 45-48 or 60-68 kDa respectively (157-161). NC can also bind to THP in the presence of calcium and magnesiumions. NC can be reversibly dissociated into its monomeric form with incubation in ethylenediaminet-etraacetic acid (EDTA) for several days. NC is composed of 110 amino acid residues of which 25% are glutamic and aspartic acid. It contains 2 cysteine and 2 or 3 γ-carboxyglutamic acid (Gla) residues which are suggested to play a significant part in its ability to inhibit CaOx crystallization. Carbohydrate content represents about 10.3% of its weight, with no glucuronic acid and 0.4% sialic acid.

Originally purified from human urine, NC has subsequently been isolated from human kidney tissue culture medium, human renal cell carcinoma, kidneys of many vertebrates, mouse renal proximal tubular cells in culture and rat kidney and urine (155, 162). Immunohistochemical techniques have localised NC in the renal epithelial cells of proximal tubules and thick ascending limb of Henle's loop (163). The site of its synthesis has not yet been confirmed by localization of NC

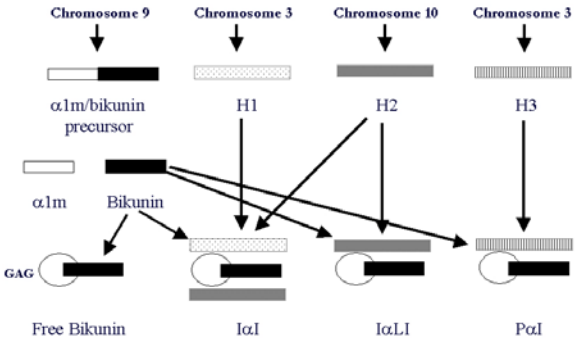

Figure 4. Schematic representation of IαI related proteins. Gene products from three different chromosomes are involved in the production of various molecules. Bikunin separates from α1microglobulin (α1m), becomes associated with chondroitin sulphate proteoglycan (GAG), is secreted free or linked to heavy chains, H1, H2, H3. Product of bikunin linking with H1 and H2 is called inter-alpha-inhibitor (IαI). Linking with H3 alone produces pre-alpha-inhibitor (PαI) while linking with H2 results in inter-alpha-inhibitor like inhibitor (IαLI). Bikunin can also link with H1 alone (not shown).

mRNA. Daily excretion of NC in human urine is about 5-16 mg (157, 158). NC was originally regarded as the principal inhibitor of CaOx monohydrate (COM) crystallization in the urine, accounting for approximately 90% of the total urinary crystallization inhibitory activity (157). According to the recent results however, the contribution of this inhibitor is suggested to be limited to only 16% (164). NC is suggested to inhibit nucleation, growth, and aggregation of COM crystals. The fractional inhibition of nucleation due to the presence of NC was shown to be equal to that of urine, (165) suggesting that this inhibitor accounts for the total nucleation inhibitory activity of urine. In an aggregation assay determined spectrophotometrically, NC exhibited inhibition of aggregation similar to THP when tested at $5X10^{-7}$M or more (166). If used at a lower concentration, NC showed less aggregation inhibitory activity than THP. The inhibitory activity of NC appeared to be reduced when ionic strength was increased, but diminished only slightly when the pH was lowered. Moreover, it was reported that this inhibitor might aggregate or bind to THP when calcium and magnesium ions were present in the milieu. NC is also suggested to participate in crystal retention by inhibiting the adhesion of COM crystals to renal epithelial cells (167).NC isolated from the urine of stone formers was structurally abnormal, and lacked Gla residues (158). Similarly the NC isolated from kidney stones also did not contain Gla residues and showed less inhibitory activity toward COM crystal formation (159). However, the amino acid composition and carbohydrate contents of NCs from both the stone formers and normals appeared similar.

Although considerable information is available regarding its pathophysiology, the amino acid sequence of NC has not yet been described and thus its identity remains to be authenticated. In this regard, Desbois *et al.* hypothesized a relationship between NC and osteocalcin (168), the most abundant noncollagenous bone protein. The osteocalcin gene cluster is composed of three genes: osteocalcin gene 1 (OG1), osteocalcin gene 2 (OG2), and osteocalcin related gene (ORG). OG1 and OG2 were expressed only in bone, whereas ORG is transcribed in kidney, but not in bone. Because ORG has a similar expression pattern and identical structure features to NC, Desbois *et al.* proposed that ORG is the gene coding for NC in the kidney. More recently (40), NC was recognised as a fragment (HI-14) of the light chain of IαI, the bikunin, on the basis of SDS-PAGE, inhibitory, and gel filtration properties and amino acid sequence of its two peptides. However the hypothesis that bikunin is NC requires confirmation due to the fact that bikunin is a part of IαI which does not contain g-carboxyglutamic acid (Gla) whereas normal NC contains 2 or 3 Gla residues. Obviously there is considerable uncertainty about the identity of NC and its contribution to crystallization inhibition, which needs to be resolved. As discussed above, NC occurs in many polymeric forms with molecular weights from 14 to 68, and it elutes from DEAE-cellulose in at least four forms.

5.2.2.3. Inter-α-Inhibitor

Inter alpha inhibitor (IαI) and related molecules collectively referred to as the IαI family, are a group of plasma protease inhibitors (169-173). These molecules are normally synthesized in the liver and are common in plasma. They are composed of a combination of heavy chains, H1 (60 kDa), H2 (70 kDa), H3 (90 kDa) covalently linked via a CS bridge to a light chain called bikunin (35-45 kDa). Separate genes located on three different chromosomes encode these chains (Figure 4). Bikunin originates from a precursor that also codes for α1-microglobulin (α1-m). The heavy and light chains also exist independently as single molecules. IαI (180-240 kDa) is a heterotrimer consisting of bikunin linked to heavy chains H1and H2. Pre-α-inhibitor (PαI, 125 kDa) is composed of bikunin and heavy chain H3. The macromolecule consisting of bikunin linked to heavy chain H2 is called IαI like inhibitor (IαLI). Bikunin is a broad-spectrum protease inhibitor and an acute-phase reactant. IαI and related proteins have been linked to various pathological conditions such as inflammatory diseases (174, 175), cancer (176-178), renal failure (179) and more recently the urinary stone disease, which we will discuss later.

In normal rat kidneys, staining for the IαI related proteins is mostly limited to the proximal tubules and generally to their luminal contents (180). We investigated renal and urinary expression of various members of the IαI family in male rats with or without experimentally induced hyperoxaluria and CaOx crystal deposition. The expression of bikunin mRNA increased in renal epithelial cells exposed to oxalate and CaOx crystals (180, 181). Eight weeks after induction of hyperoxaluria, various sections of renal tubules stained positive for IαI, bikunin as well as H3. Positive staining was observed in both the tubular lumina as well as cytoplasm of the epithelial cells. Crystal associated material was heavily stained. Western blot analysis of urinary proteins recognised 7 bands. Urinary expression of H1, H3 and pre-α-inhibitor was significantly increased. Tissue culture studies have shown that human renal proximal tubular epithelial cells constitutively express genes for bikunin and H3 components (182). Bikunin gene

is also expressed in MDCK cells and was upregulated when they were exposed to oxalate (183).

Both heavy and light chains have been identified in the urine (184-199). The average concentration of IαI in the plasma of healthy human subjects is approximately 450 mg/l (196). Urinary excretion is 2 to 10 mg/day but can increase to 50-100 fold or more in certain pathological conditions such as cancer. Plasma concentration of IαI is on the other hand reduced during various pathological conditions including renal failure. Several investigators have determined the concentration of bikunin in the normal human urine. Some of the published values are: 0.225-0.650 μg/ml (191), 5.01 + 0.91 μg/ml (190), 10.13 + 1.13 μg/ml and 6.72 + 0.93 μg/ml in normal men and women respectively (192), 4.82 + 2.46 mg/day and 3.86 + 1.35 mg/day in normal men and women respectively (193), and 17.5 mg/day (197). It has been suggested that most of these values are overestimation since quantification employed immunoassays using polyclonal antibodies against bikunin. Such antibodies will crossreact with all bikunin containing members of IαI family and thus actually overestimate the concentration of free bikunin in the samples. As we mentioned above, bikunin excretion is generally increased in the urine of patients with renal disease. As a result, an increase in CaOx inhibitory activity is anticipated. However, this was not the case, suggesting that bikunin obtained from stone formers may be structurally abnormal. It was shown that bikunin isolated from the patients, contained less sialic acid and exhibited less crystallization inhibitory activity than that purified from the urine of healthy subject (188). In a separate study mean urinary bikunin to creatinin ratio was found to be significantly higher in stone formers than in non-stone forming healthy male and female controls (189). Western analysis showed that a significantly higher proportion of stone patients had a 25kDa bikunin in their urine in addition to the normal 40kDa species. 25kDa bikunin was similar to the deglycosylated bikunin and was less inhibitory. Yet another study found decreased urinary excretion of bikunin by stone forming patients. Mean urinary excretion of bikunin in 18 healthy individuals was 5.01 + 0.91 μg/ml and 2.54 + 0.42 μg/ml (P=007) in 31 stone patients (190).

With respect to kidney stone formation, Atmani *et al.* isolated a 35kDa urinary protein, which inhibited growth of CaOx crystals. They named the protein uronic acid rich protein (UAP), because of the high uronic acid content with D-glucuronic and L-iduronic acids being the major constituents (184). Amino acid composition revealed it to be rich in aspartic and glutamic acid residues, which account for 24% of the total amino acids. No Gla residues were detected. Basic and aromatic amino acids represented 10% and 13%. Carbohydrates accounted for 8.5% of its weight. N-terminal amino acid sequence analysis of human protein demonstrated the homology with IαI, specifically with bikunin (185). Later we isolated the UAP from the rat urine (186) and showed it to have characteristics similar to the human UAP in molecular weight, amino acid composition as well as the crystallization inhibitory activity. In addition, on Western blot analysis, both reacted with an inter-α-trypsin inhibitor antibody. Later, on the basis of bikunin antibody reaction with the UAP in western blot analysis and similarity of the sequence of first 25 N-terminal amino acid residues of UAP being identical to that of bikunin we identified the UAP as bikunin (187).

IαI proteins have been shown to inhibit CaOx crystallization *in vitro* (184-187, 190, 191, 195, 197). The inhibitory activity is confined to the carboxy terminal of the bikunin fragment of IαI (197). Both rat and human urinary bikunin inhibited nucleation and growth of CaOx crystals. Treatment with chondroitinase AC had no effect on this inhibitory activity, which was destroyed by pronase treatment indicating that the activity lies not with the chondroitin chain but with the peptide. In an *in vitro* experiment nucleation and aggregation of CaOx crystals were studied by measuring turbidity at 620 nm (198). Crystallization was induced by mixing calcium chloride and sodium oxalate at final concentrations of 3 and 0.5 mM, respectively. Both solutions were buffered with 0.05 M Tris, 0.15 M NaCl, pH 6.5. Nucleation measurements were performed at 37˚C, with stirring at 800 rpm. Inhibition of nucleation was estimated by comparing the induction time in the presence or absence of the inhibitor. In the aggregation assay, the optical density of the solution containing CaOx monohydrate crystals was monitored. Inhibition of aggregation was evaluated by comparing the turbidity slope in the presence of the inhibitor with control values. The data showed that urinary bikunin, at concentrations of 2.5 to 20 μg/ml, retarded crystal nucleation by 67 to 58% and inhibited crystal aggregation by 59 to 80%. These results were confirmed later when inhibition of CaOx crystal growth and aggregation by IαI, its heavy chains, light chain (bikunin) with or without chondroitinase treatment, and bikunin's carboxy terminal domain (HI 8) was tested in an *in vitro* crystallization assay (197). IαI was a weak inhibitor while heavy chains showed no discernible activity. Bikunin and HI effectively inhibited the crystallization. Chondroitinase treatment had no effect on the inhibitory activity of bikunin. IαI molecule itself is also an effective inhibitor of CaOx crystal growth (187) and in a recent study was shown to be more efficient than another crystal growth inhibitor, prothrombin fragment-1 (191).

Bikunin has also been implicated in modulating adhesion of CaOx crystals to the renal epithelial cells (199). MDCK cells were exposed in culture to CaOx monohydrate crystals in the presence or absence of various protein fractions isolated from normal human urine. A single fraction with a molecular weight of 35 kDa was found to be most inhibitory of crystal adhesion. This protein inhibited crystal adhesion at the minimum concentration of 10ng/ml and completely blocked it at 200ng/ml. Amino acid sequence of the first 20 amino acids of the N-terminal was structurally homologous with bikunin.

α1-microglobulin (α1-m) is also an inhibitor of CaOx crystallization *in vitro* (200). α1-m was isolated from human urine. Two species of 30 and 60 kDa, recognized by the antibody against α1-m, were isolated. Both inhibited CaOx crystallization in a dose dependent manner. Using an ELISA assay, urinary concentration of α1-m was found to

be significantly lower in 31 CaOx stone formers than in 18 healthy subjects (2.95 + 0.29 vs. 5.34 + 1.08 mg/l respectively, P = 0.01).

Recent studies have provided evidence that CaOx stone-forming men excrete significantly more IαI and IαLI in their urine than age and race matched non-stone-forming men (201). The increased expression was not limited to CaOx stone forming males. Uric acid stone formers demonstrated similar tendencies. In contrast high molecular weight IαI related proteins were common in both stone forming and non-stone-forming women. When urinary proteins were resolved by SDS-PAGE, the relative density of IαI was approximately threefold greater in females than in males (202). Male children (< 10 years) excreted 2-7 fold higher amounts of the high molecular weight IαI than the adult males (203). Since stones are much more common in adult males than in females, it was argued that perhaps urinary excretion of these high molecular weight IαI macromolecules was somehow responsible for the difference and that it was influenced by the sex hormones. However a comparison between normal male adults, male adults undergoing androgen deprivation and or postmenopausal females on estrogen therapy showed that there were no differences in the relative levels of urinary IαI among various groups of age and sex matched individuals (203).

5.2.2.4. Osteopontin

Diverse functions and widespread distribution of osteopontin (OPN) have recently been reviewed (17, 204, 205). OPN is a noncollagenous phosphoprotein originally isolated from mineralized bone matrix where it is made by osteoblasts. Its apparent molecular weight has been estimated from 44 to 75 kDa depending on the percentage of polyacrylamide gel used. This anomalous migration is assumed to be due to differences in glycosylation and phosphorylation. In addition to its existence as a monomeric form, the protein may also aggregate to form a higher molecular weight entity. Amino acid analysis of rat OP revealed that it contains 319 residues of which 36% are aspartic and glutamic acid (204, 206). It also contains 30 serine, 12 phosphoserine and one phosphothreonine residues. None of the glutamic acid residues are γ-carboxylated. The carbohydrate content represents 16.6% with the presence of 10 sialic acid residues per molecule. The presence of mannose and N-acetylgalactose suggests an N and O-linked oligosaccharides respectively. One interesting part in the structure of OPN is the identification of the sequence Arg- Gly-Asp (RGD), which is presumed to be involved in cell attachment via α v β 3 integrin receptors. In close proximity to the RGD region is a thrombin cleavage site. Thrombin cleaves OPN into 2 fragments, an amino (N)-terminal fragment with RGD sequence and a carboxyl (C)-terminal fragment. OPN affects cell functions through its receptors, the members of integrin and CD 44 families (207, 208). The thrombin cleavage of OPN allows for greater access of the RGD domain to the receptor sites. Osteopontin from all species has high aspartate/asparagine contents accounting for as much as 16-20% of all amino acid residues in the molecule. This highly negatively charged molecule could chelate 50 calcium ions/ molecule of protein (209).

In addition to bone cells, OPN is present in many epithelial tissues in kidneys, gastrointestinal tract, gall bladder, pancreas, lung, salivary gland and inner ear (205). It is also expressed in a variety of other cell types including macrophages (210, 211), activated T-cells, smooth muscle cells and endothelial cells. Regulation of OPN expression, synthesis and production is incompletely understood but is considered to be controlled by a variety of factors such as parathyroid hormone, vitamin D, CaP, various growth factors, cytokines, sex hormones and a variety of drugs (17, 204, 205). For example mediators of acute inflammation such as tumor necrosis factor-α (TNFα) (212) and interleukin-1β (213) induce OPN expression. Other mediators that can induce OPN expression are angiotensin-II and transforming growth factor-β (TGF-β). OPN expression is enhanced in the injury and recovery processes including inflammation, fibrosis, mineralisation and regeneration.

Localization studies in mouse kidney by immunohistochemistry and *in situ* hybridization have shown that the expression of OPN is somewhat heterogeneous (214, 215). OPN was detected in thick and thin ascending limb of the loop of Henle and distal convoluted tubules and macula densa. It was prominent along the apical surface of the cells lining the lumen. The expression of the protein becomes stronger in pregnant or lactating female mice (215). In ageing mice this expression was extended from its normal distal locations to proximal locations including glomeruli. The expression of OPN mRNA was not detected in proximal tubules, thin descending limbs, collecting ducts or glomeruli. Recent studies in rats have shown that OPN is localised to thin limbs of the loop of Henle as well as the papillary surface epithelium in the calyceal fornix (46, 216). In normal human kidneys OPN is localised primarily to the thick ascending limb of the Henle's Loop and distal convoluted tubules (217). Apparently the expression of OPN in normal kidneys is species, age and gender dependent.

The mean OPN excretion for normal humans varies between 2.4 and 3.7 mg/day (218) or 1.9μg/ml (219), is inversely related to urinary volume and is not affected by urinary excretion of calcium. Urinary OPN was originally isolated as a glycoprotein with a molecular weight of approximately 50 kDa on 16% SDS-polyacrylamide gel. It showed a structural homology with OPN and was named uropontin based on its origin from kidney and presence in the urine (220). Stone formers excrete less OPN than non-stone formers, presumably because of the incorporation of OPN in crystals of the growing stone (221).

The significantly higher incidence of a single base mutation in the OPN gene has been found in the patients with recurrent or familial nephrolithiasis (222). OPN is intimately involved in both the physiological and pathological mineralisation processes including crystallization in the urine and development of calcific kidney stones.

Experiments with hyperoxaluric rats have provided convincing evidence for an association between CaOx crystal deposition and OPN expression. Experimentally induced hyperoxaluria is almost always associated with increased epithelial expression of OPN (46, 154, 223), that co-localises with CaOx crystal deposits (46, 223). In a recent study hyperoxaluria and CaOx crystal deposition was induced in male Sprague-Dawley rats. Immunohistochemical localisation, *in situ* hybridization and reverse transcriptase quantitative competitive polymerase chain reaction (RTPCR) were employed to examine OPN expression in the kidneys. Urinary excretion of OPN was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and densitometric analysis of the western blots. OPN expression increased significantly after hyperoxaluria and increased even further after crystal deposition (224). Urinary excretion concomitantly increased. Although there was an increase in the expression of OPN during hyperoxaluria, it was primarily limited to cells of the thin loops of Henle and the papillary surface epithelium. However after crystal deposition OPN was expressed throughout the kidneys including segments of the proximal tubules. A number of compounds such as citrate (225), allopurinol (226), and female sex hormones (227) have been shown to reduce CaOx crystal deposition in kidneys of hyperoxaluric rats. Administration of these compounds also reduced OPN expression in the hyperoxaluric rats leading the authors to conclude that reduction in crystal deposition may also be a result of down regulation of OPN synthesis and secretion.

Support for the concept of oxalate and CaOx crystal induced up regulation of OPN is also derived from tissue culture studies. Exposure of MDCK or BSC-1 cells to CaOx monohydrate crystals stimulated the production of OPN (228). Long-term cultures of MDCK produce CaP deposits that contain osteopontin and calprotectin also when the MDCK cells are transplanted into nude mice (229, 230). When human renal proximal tubular epithelial cells were exposed to 1mM total oxalate there was a significant increase in OPN mRNA (231). OPN is a potent inhibitor of nucleation (165, 232), growth and aggregation of CaOx crystals (233). OPN is also a regulator of crystal adherence to cell surfaces, which may lead to crystal retention within the kidneys, an important aspect of stone formation.

Recently, it was demonstrated that OPN also inhibits the adhesion of CaOx monohydrate as well brushite crystals to BSC-1 renal epithelial cells in culture (59). Adhesion inhibition was accomplished by coating the crystal surfaces and not the cell surfaces. Interestingly OPN has no effect on adhesion of uric acid crystals in similar assays using BSC-1 renal epithelial cell line. Other studies have however, shown that OPN promotes adhesion of CaOx crystals to the renal epithelial cells. Exposure of MDCK cells to CaOx crystals in the presence of added OPN promoted crystal adherence to the cells (234). On the other hand removal of OPN from the culture medium by adding OPN polyclonal antibody, thrombin, cyclic Arg-Gly-Asp peptides or tunicamycin inhibited crystal adherence by 80%, 50-80%, 60-80%, or 50-60% respectively. Inhibition of OPN synthesis by NRK-52E cells transfected with antisense OPN expression vector resulted in decreased adherence of CaOx crystals (235). Similarly inhibition of OPN production by MDCK cells by introducing antisense oligonucleotide also resulted in decreased crystal adherence (236).

Support for the CaOx crystallization inhibitory actions of OPN is further strengthened by studies in OPN knockout mice (237). When comparable hyperoxaluria is induced in OPN knockout and wild type mice, knockout mice developed significant intratubular deposition of CaOx crystals while wild type remained free of any crystals. In addition wild type hyperoxaluric mice showed significant increase in OPN expression in their kidneys, indicating a reno-protective role for OPN. Results of one study show OPN favouring crystallization of COD over COM (238), which may influence the development of kidney stones because renal epithelium is more likely to bind COM crystals than the COD crystals. It appears that structural defects, and various post-translational modifications, such as glycosylation and phosphorylation may influence the effect of OPN on crystallization in urine.

5.2.2.5. Urinary Prothrombin Fragment –1(UPTF-1)

A protein with a molecular weight of 31 kDa was found selectively associated with CaOx crystals experimentally induced in human urine (239). This protein was called crystal matrix protein (CMP). The amino acid sequence analysis of CMP showed an identity with prothrombin (240-242), a plasma protein involved in coagulation cascade. In the presence of different activation factors, prothrombin (PT) is subjected to a series of cleavages releasing small fragments including fragment 1 (F1) and F1+2. Prothrombin as well as fragments 1 and 2 has been detected in the urine. Suzuki *et al.* proposed that CMP is the activation peptide of human prothrombin (242). By using specific antibodies for prothrombin and F1+2 fragment, Stapleton and Ryall demonstrated (241) that CMP is prothrombin fragment F1 (UPTF-1). The mean value of daily urinary fragment F1 excretion is 13.4 nM/day as estimated by using radioimmunoassay technique (243). Excretion is increased in pregnant women to 47.2 nM/day. F1 fragment contains about 154 amino acids of which 23% are glutamic and aspartic acids (244). In the first 34 amino acid residues, 10 of the glutamic acids are γ-carboxylated. The carbohydrate contents represent 17% of its molecular weight.

Anderson and co-worker reported the localisation of PT exclusively in the liver (245). Stapleton *et al.* using a polyclonal antibody found a positive reaction in the kidneys (246). The staining was localized in cytoplasm of the epithelial cells of thick ascending limb of the loop of Henle and the distal convoluted tubules including the macula densa. The amount of F1 was significantly increased in the kidneys of stone patients compared to those in healthy subjects (247). Recent studies have provided evidence that PT gene is expressed in both the human and rat kidneys indicating the possibility of PT biosynthesis in both human and rat kidneys (248-250). Recent studies using purified

urinary proteins have confirmed earlier results and have demonstrated UPTF-1 to be an inhibitor of both crystal growth and aggregation (251). Results of another study where a comparison was being made between the white and black South Africans with regard to urinary crystallization inhibition showed that UPTF-1 is a strong inhibitor of crystal nucleation (252). UPTF-1 from normal black males reduced crystal nucleation by 63.6% as compared to the protein from normal white males that reduced the nucleation by 23.4%. When crystallization inhibitory potential of all the prothrombin related peptides, the PT, thrombin (T), and fragments 1 and 2 was tested using a simple inorganic solution, both prothrombin and fragment 1 were found to inhibit crystal aggregation (253). Various peptides reduced the size of aggregates in the order F1>PT>F2>T. However when similar experiments were conducted using undiluted, centrifuged and ultrafiltered human urine (254), crystal aggregation was inhibited only by PT fragment-1.

The crystallization inhibitory activity is dependent on the Gla domain, which is absent from thrombin and F2, and both PT as well as F1 fragments include (254). Why is F1 more potent than PT? Probably as a result of F1's greater charge to mass ratio. Prothrombin has the same number of Gla residues as F1 but a molecular weight of 72 kDa, which is more than double that of F1 at approximately 31 kDa. Prothrombin's isoelectric point is also higher.

5.2.2.6. Calgranulin (Calprotectin)

Calgranulin is a 28 kDa member of S100 family of calcium binding proteins, which are small, ubiquitous, and acidic proteins involved in normal developmental and structural activities (255). However they are also implicated in a number of diseases (256). The protein was recently isolated from human urine (257) at a concentration of 3.5-10 nM. Purified urinary calgranulin inhibited both CaOx crystal growth (44%) and aggregation (50%) in nanomolar range. 28kDa calgranulin was cloned from the human kidney expression library. Western analysis of rat and human kidneys as well as renal epithelial cell lines, BSC-1 and MDCK confirmed its renal presence. Calgranulin is also known as leukocyte antigen L1 and has been identified in circulating neutrophils and 22 monocytes and has bacteriostatic antifungal activites (258). It has also been identified in matrix of infectious or struvite stones (259) and in CaP deposits formed by MDCK cells (229, 230).

5.2.2.7. Albumin

Albumin is one of the most abundant proteins in the urine (45, 260, 261) and has been detected in the matrix of both urinary stones (260-262) as well as crystals (42, 43, 263) made in the whole human urine. It is known to bind to CaOx as well as uric acid crystals (264, 265) but does not inhibit their growth (145, 264). However it has been shown to inhibit CaOx crystal aggregation in concentration dependent manner (266-268). When immobilized to surfaces and exposed to metastable solutions albumin promotes crystal nucleation (76, 269). When dissolved in solution albumin exists either in monomeric or and polymeric form (76). In metastable CaOx solutions both monomeric and polymeric forms promote nucleation of CaOx. In addition, nucleation by albumin leads exclusively to the formation of COD crystals. Urinary albumin purified from healthy subjects contained significantly more polymeric forms and was a stronger promoter of CaOx nucleation than albumin from idiopathic calcium stone formers. Promotion of CaOx nucleation and formation of large number of COD crystals might be protective. Nucleation of large number of small crystals would allow their easy elimination and decrease CaOx saturation preventing crystal growth and aggregation and subsequent stone formation. COD crystals are more common than COM crystals in non-stone formers urine and are generally found in lesser quantities in stones than COM crystals. In addition crystals present in the urine from non-stone formers are significantly smaller than those in stone formers urine.

Albumin also exhibits the capacity to bind some of the urinary proteins. Interestingly, urinary proteins that show great affinity for albumin are also those that are included in the stone matrix. It is suggested that proteins become a part of stone matrix by binding to the albumin coating CaOx crystals. It is also suggested that unlike other calcium binding urinary proteins, albumin promotes nucleation by interacting with calcium through the carboxyl group. Strong nucleation activity was observed at pH 7 but was totally eliminated at pH 4 when carboxyl groups are no longer ionized. In addition, morphological studies showed CaOx crystals to nucleate through calcium rich face (76).

5.2.2.8. CD44

CD44 is a transmembrane protein and the main cell surface receptor for hyaluronan or hyaluronic acid (HA) as well as OPN (208). Both CD44 and HA are upregulated during injury and inflammtion and are involved in the formation of a cell coat or pericellular matrix on surfaces of proliferating and migrating cells. HA is restricted to the inner medullary interstitium of the normal kidneys. Distal collecting duct cells express both CD44 and HA on apical cell surfaces of the proliferating cells. At confluence however, CD44 is expressed at the basolateral membrane while HA is undetectable. Proliferating cells are receptive to adhesion of CaOx crystals, a property lost when cells become confluent. In addition removal of pericellular matrix by hyaluronidase treatment also results in loss of crystal adhesion property of the proliferating cells (319, 320). Based on theses observations it has been proposed that intact epithelium does not bind crystals because of the absence of a pericellular matrix and crystal attachment depends upon the expression of CD44, OPN and HA by the damaged renal epithelial cells (88).

5.2.2.9. Model Peptides

A number of studies have been carried out investigating the effect of model peptides on crystallization *in vitr*o. Polyaspartic acid (PolyD) with molecular weights of 8, 12, 15, 37.6 and ploy glutamic acids (PolyE) with molecular weight of 13 have been examined. A clear understanding of the crystallization inhibitory mechanisms of various glycoproteins has been the main purpose of these studies. Crystallization of CaOx was induced *in vitro* in a buffered salt solution containing calcium and oxalate in different ratios and at

various supersaturations, in the absence or presence of the polypeptides with pH and ionic strength in the range of normal human urine (238, 270, 271). In the absence of proteins, CaOx monohydrate was the preferred crystalline form for all Ca to Ox ratios (270, 271). The number of CaOx monohydrate crystals increased with increasing oxalate concentrations. The presence of either the Poly D or E produced COD crystals. PolyE was less effective at producing COD than PolyD (271). At a concentration of 800nM and equimolar Ca and Ox concentrations only 20% of the crystals were COD's. It did however have an effect on COM crystal morphology by producing dumbbell shaped crystals, a morphology common in human and rat urine. Under similar conditions of supersaturation and Ca and Ox concentrations PolyD, however, favoured the formation of COD requiring very low concentrations <200nM. 12, 15 23 and 37.6 molecular weight PolyD were able to exclusively produce almost all COD's. Higher CaOx supersaturations required higher amounts of PolyD to cause COD formation.

It is concluded that change from COM to COD is the result of inhibition of COM nucleation by protein adsorption onto nascent nuclei. COD is formed to relieve the chemical potential favouring crystallization. The importance of these results with regard to nephrolithiasis lies in the observations that COD crystals are less likely to adhere to the renal epithelium than COM crystals and thus, less likely to be retained in the kidneys and promote the formation of kidney stones (271). Both PolyD and PolyE have also been tested for their effect upon COM crystal growth and adherence to renal epithelial cells in culture. Both proved potent inhibitors of the growth of COM crystals and also blocked the adhesion of COM to BSC-1 cells (59).

5.3. Lipids And Cellular Membranes

The role of cellular membranes and their lipids in both physiological and pathological calcification is well-established (272-274). According to current concepts, initial CaP deposition in a number of calcific diseases occurs on cellular membranes, which are present at the calcification sites either as a limiting membrane of the so called matrix vesicles (273, 274) or as cellular degradation products (272). One of the main reasons that cellular membranes act as specific nucleators of CaP is proposed to be the presence of lipids and in particular the acidic phospholipids. Lipids have been demonstrated both histochemically and biochemically at physiological as well as pathological calcification sites. *In vitr*o, membranes, acidic phospholipids, lipid extracts from various calcified tissues, as well as lipid containing liposomes have been shown to initiate CaP formation from metastable solutions.

Small amounts of lipids appear in urine under normal circumstances, while urinary excretion of cholesterol, phospholipids, triglycerides and free fatty acids is increased in many diseases (275). Many of the lipids may be of membrane origin since epithelial cells are continuously sloughed and excreted by both normal human males and females (276). Biochemical analysis discloses that urine from stone formers contain more and different phospholipids than that from normal humans (277). Human urine is metastable with respect to the common calcific crystals, CaOx and CaP; i.e. urine would require a substrate or a nucleation platform for crystallization to occur. Membrane vesicles and fragments with their lipids provide a suitable site for crystal nucleation. Membranous vesicles obtained from the brush border of the rat renal tubular epithelium (278) and lipid components of the human stone matrix are good nucleators of CaOx from an inorganic metastable solution (279). When CaOx crystallization was induced in whole human urine by the addition of oxalate, CaOx crystals appeared in association with the cellular membranes and almost all phospholipids present in the urine became a part of the crystal matrix (33).

To verify that cellular membranes present in the urine promote nucleation of CaOx we removed these urinary constituents by filtration or centrifugation and induced crystallization by adding oxalate before and after filtration or centrifugation (280). We also performed reconstitution studies in which substances such as total retentate, proteins, total lipids, neutral lipids and phospholipids, which are removed during filtration, were added back into the filtered urine prior to induction of nucleation. In addition, we examined CaOx crystallization after membrane vesicles isolated from rat renal tubular brush border were introduced into the filtered or centrifuged urine. We also determined urinary metastable limit with respect to CaOx. Both filtration and centrifugation resulted in a significant increase in metastable limit, which was reduced by the addition of membrane vesicles. Filtration resulted in a significant reduction in the number of crystals formed. A highly significant increase occurred when various components were added back into the filtered urine. The highest and most significant increase occurred when phospholipids were added back into the filtered urine.

To understand crystallization in the kidneys one has to appreciate the following: 1, urine spends only a few minutes (approximately 3 minutes) in the renal tubules (12, 281) and only seconds in various segments (2); 2, urinary composition, pH and supersaturation with regard to CaOx and CaP changes as urine courses through the renal tubules(281-283). Urine in the loop of Henle can support nucleation of CaP while in the distal tubules and collecting ducts urine is susceptible to nucleation of CaOx. With this in mind, we studied crystallization of CaOx *in vitro* (282). Nucleation was allowed to occur *in vitro* in solutions with ionic concentrations simulating urine in various segments of the renal tubules namely proximal tubules, descending and ascending limbs of the loop of Henle, distal tubules and collecting ducts. A constant composition system was used and experiments were run for 2 hours with or without the added renal brush border membrane vesicles (BBM). The addition of BBM significantly reduced the nucleation lag time and increased the rate of crystallization. The average nucleation lag time decreased from 84.6 ± 43.4 minutes to 24.5 ± 19 minutes in proximal tubule urine, from 143.6 ± 29 to 70.2 ± 53.4 minutes in descending limb of the loop of Henle urine, from 17.6 ± 8.6 minutes to 0.625 ± 0.65 minutes in distal tubules urine, and from 9.54 ± 3.03

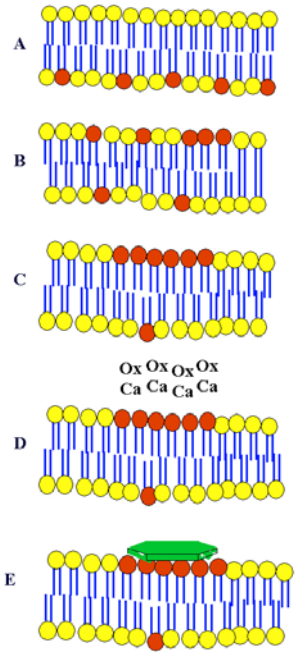

Figure 5. Schematic presentation of changes in cell membrane phospholipids and nucleation of calcium oxalate crystals based on Khan *et al.* (284), Backov *et al.* (289) and Wiessner *et al.* (291). A, Normal membrane with only the neutral phospholipids (yellow circles) on the outer surface. B, Movement of acidic phospholipids (red circles) from inside to the outside. C, Lateral movement of acidic phospholipids into specific domains. D, Concentration of calcium ions and their interaction with membrane phospholipids on one side and with oxalate ions on the other side. E, Nucleation of a CaOx crystal

minutes to 0.625 ± 0.65 minutes in collecting duct urine. There was no nucleation without BBM in the urine from ascending limb of the Henle's loop. COD was common in most solutions. CaP also nucleated in the urine in descending limb of the loop and collecting duct. In the absence of BBM there was no crystallization in any of the solutions within the time frame that urine generally spends in the renal tubules indicating the unlikelihood of homogeneous crystal nucleation in the renal tubules. In addition, whole urine contains many crystallization inhibitors, which should make it even more difficult. However BBM-supported nucleation is likely in both distal tubules and collecting ducts because in these segments nucleation lag time for CaOx was much shorter than the urine residence time.

It is suggested that anionic head groups of membrane lipids when exposed to urine attract Ca^{2+} ions. A cluster of sufficient number of properly aligned Ca^{2+} ions can then interact with oxalate (Figure 5) or phosphate leading to nucleation of CaOx or CaP crystals (284, 285). In vitro crystallization studies with Langmuir monolayers of phospholipids have demonstrated selective nucleation of CaOx monohydrate crystals with the (10-1) calcium rich face towards the monolayer (286-289). Normal membranes do not expose many anionic head groups on their surfaces. Catastrophic events can however, induce both lateral and trans-membrane migration of phospholipids and sequester them in specific domains (290). Exposure to high levels of calcium and oxalate, a most likely event since increased urinary excretion of oxalate and calcium is common in stone formers, induces several changes in the renal epithelial cell membrane. Oxalate and CaOx crystals induce redistribution of PS from the inner leaflet of the membrane to the outside (291-293) and high levels of calcium (294) partition them in specific domains. Vesicles derived from these membranes would be capable of supporting crystallization in the urine metastable for CaP and CaOx.

Altered membrane properties that support crystal nucleation can also provide for crystal attachment to the renal epithelium (290) as well as crystal aggregation (11, 285). Crystal binding to membrane fragments flowing in the urine would promote crystal aggregation. This is evident in vivo from the observations that urine from hyperoxaluric rat shows crystals aggregating around and associated with membrane fragments (11). Binding of crystals with their calcium rich face to negatively charged cell membrane of the renal epithelium would possibly promote crystal adhesion and retention within the renal tubules. Phosphatidylserine (PS) has been thoroughly studied as a crystal adhesion molecule on cell surfaces. There was a dose dependent increase in the cell surface PS when continuous cultures of the inner medullary collecting duct were exposed to oxalate (290, 291). Increased surface expression of PS correlated with a corresponding increase in CaOx monohydrate crystal attachment to the cells. Crystal attachment was inhibited by treatment with Annexin V, a protein that specifically binds to PS. In addition insertion of PS into the cell membranes promoted crystal attachment. The possibility of PS associated attachment of CaOx crystals is highly feasible in the kidneys of stone formers. Renal epithelial cells exposed to high levels of oxalate and/or CaOx crystals undergo apoptosis (292) in which PS moves from inside to outside of the membrane and thus becomes exposed to the urinary environment.

Further evidence of the role of cellular membrane constituents in urolithiasis is provided by investigations of human and experimental nephrolithiasis. Experimental studies of CaOx urolithiasis induced in laboratory rats have shown that irrespective of the nature of the hyperoxaluria inducing agent used, the administration protocol utilised, or the location of crystal deposition within the urinary system, CaOx crystals were always found associated with cellular degradation products consisting mostly of membrane bound vesicles (11, 295, 296). Even when a foreign body was implanted in the urinary bladder of hyperoxaluric rats, crystals did not nucleate on the native foreign body but on the membranous organic material that coated the foreign body surface (297). Intranephronic calculosis in the laboratory rat caused by feeding purified diets is membrane mediated (298, 299). Vesicles derived from the microvillous brush border of the proximal tubules provide the nidus for intratubular deposition of CaP. The deposits start in the lumens of segment I of the proximal tubule and travel down the nephron while at the same time accrue

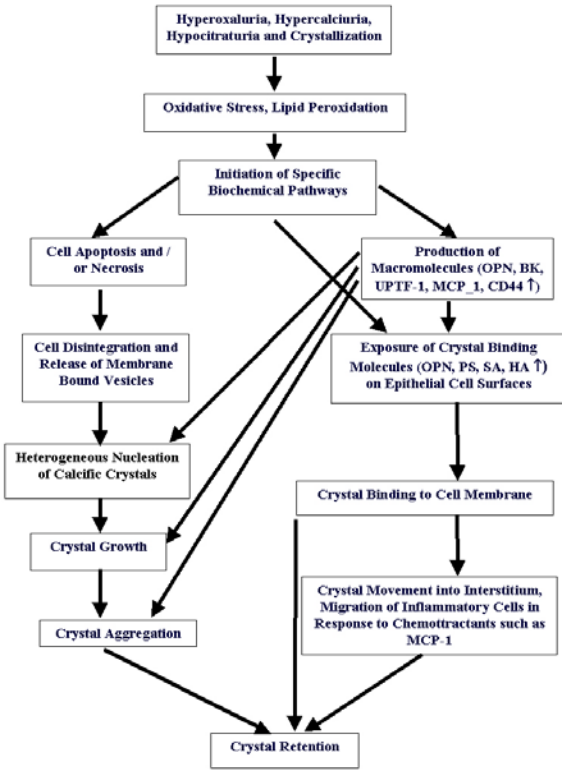

Figure 6. Schematic representation of cellular and extracellular events during calcium oxalate nephrolithiasis. OPN, osteopontin; BK, bikunin; UPTF-1, urinary prothrombin fragment-1; PS, phosphatidylserine; SA, sialic acid; HA, hyaluronic acid; MCP-1, monocyte chemoattractant-1.

mass by laminar growth in which both membranous vesicles and mineral are involved. Finally these microliths become large enough to be retained at the junction of the proximal tubule and the loop of Henle. The mineral content of the microliths increases with the result that they become much more compact and the laminated structure is finally lost.

In the case of human nephrolithiasis, a number of renal stones start at the tips of renal papillae on sites called Randall's plaques (300, 301). In a recent study these plaques appeared to be the stone nidus especially in stoneformers with a normal oxalate excretion but increased calciuria (14). In that study the plaques appeared to start extratubular. The plaques contain cellular degradation products and crystals of CaP. Stones formed on such plaques, were once thought to be rare but a study of five hundred spontaneously passed small stones, collected in Spain by Cifuentes-Delatte, has shown that 142 (28.4%) of them contained nidi of necrotic material identifiable as calcified tips of the renal papillae (302). The results indicate that at least in these cases the stones could have originated by heterogeneous nucleation of crystals on the membranous cellular degradation products.

6. SUMMARY

It has been well established that there are significant differences between normal and stone formers with respect to the excretion of various participating ions such as calcium, oxalate, and citrate as well as crystallization modulating macromolecules. Only recently has it been shown that this altered urinary milieu has a considerable effect on the renal epithelial cells and it is the cellular response which determines whether localized supersaturation with respect to various crystals will result in crystalluria or lead to stone formation. Under normal circumstances, renal epithelial cells internalize the occasional crystals, proliferate, and attempt to dissolve them in their lysosomes or externalize them into the interstitium to be handled by the inflammatory cells. Beset with conditions of high calcium and/or oxalate and calcium phosphate and/or CaOx crystals, however, the cells are injured and go through apoptosis and/or necrosis initiating a cascade of events leading to further crystallization, crystal retention and formation of stone nidi.

Results of both the animal model and tissue culture studies indicate that renal cells respond by increased production of various modulating (inhibitory) macromolecules. This is contrary to the clinical observations that stone formers excrete less inhibitors and their urine is less inhibitory. The answer to this dilemma perhaps lies in the observation that kidney stones contain a pervasive matrix made of a variety of excreted macromolecules. Thus despite increased production, urinary excretion of macromolecules appear lower. Almost all the crystallization modulating macromolecules discussed here are also prominent in cell injury, inflammation and recovery. In addition some of the inflammatory molecules such as MCP-1 with no apparent role in crystallization are also produced by renal epithelial cells on exposure to oxalate and CaOx crystals indicating a close relationship between inflammation and nephrolithiasis (17, 180, 224, 321).

Most of the inhibitory molecules are anionic, with many acidic amino acid residues, frequently contain post-translational modifications such as phosphorylation and glycosylation, and appear to exert their effects by binding to CaOx surface. In a number of cases, stone patients' urine exhibit abnormalities of protein structure or function. It is not yet known what proportion of stone formers have an abnormality of inhibitor function. The specific structural motifs that favour crystal nucleation, binding and inhibition are not yet fully understood. Lipid headgroups appear to be involved in crystal nucleation. A number of the proteins and glycosaminoglycans are produced in the kidneys. Some glycosaminoglycans are added to the urine in the bladder. Still others gain access by glomerular filtration. The presence of lipids in the urine is an outcome of death and degradation of renal epithelial cells caused by exposure to high levels of urinary oxalate and CaOx crystals. Further information on regulation of expression of these macromolecules and their activities in the specific microenvironment may provide new avenues for the prevention and recurrence of stone formation.

Figure 6, provides a schematic presentation of the events during CaOx nephrolithiasis and the production and involvement of various modulators in crystallization and

urinary stone formation. Oxalate and CaOx crystals interact with renal epithelial cells and induce oxidative stress. On the one hand it may cause lipid peroxidation and apoptotic and/or necrotic cell disintegration releasing membrane vesicles into the milieu. Membranes of the cellular degradation products promote nucleation of the calcific crystals. Oxidative stress may also induce signal transduction (322-323), initiate specific biochemical pathways and gene expression leading to the production of crystallization modulators such as OPN, BK, CD44 and UPTF-1, which influence crystal nucleation, growth, aggregation and/or retention. Oxidative stress may also be responsible for the exposure on epithelial cell surfaces of specific crystal binding molecules such as phosphatidylserine, sialic acid or hyaluronic acid. Adherent crystals may be endocytosed and moved into the interstitium where crystals become surrounded by monocytes and macrophages in response to the release of chemoattractants such as MCP-1 by the oxalate exposed renal epithelium.

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