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The dual effect of urinary macromolecules on the crystallization of calcium oxalate endogenous in urine

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Summary. The nucleation-promoting and growth-inhibiting activities of urinary macromolecules on the crystallization of calcium oxalate endogenous in urine of stone-formers and normal controls were studied by freezing the ultrafiltrate and retentate fractions of concentrated whole urine (pH 5.3, 1,250 mosmol/kg). Among the normal controls, macromolecules nominally of 10–20 kDa showed nucleation-promoting and growth-inhibiting activities; the 5–10 kDa population was incapable of such effects but did cooperate with molecules >10 kDa in enhancing the effect. In the case of stone-formers, molecules in the nominal ranges of 5–10 kDa and 10–20 kDa when considered separately were not active in the aspects studied but collectively could cooperate to produce pronounced effects. Application of the test to urine ultrafiltrate reconstituted with polyanionic macromolecules recovered from urine indicated that molecules from stone-formers were more powerful than those from normal controls in bringing about promotion of nucleation and inhibition of growth of crystals from urinary calcium oxalate.

Key words: Crystallization – Calcium oxalate – Macromolecules – Urinary polyanions – Ultrafiltration

Studies in search of a pathogenetic role for the organic matrix in idiopathic calcium urolithiasis have disclosed diverse activities of urinary macromolecules in regard to crystallization of calcium oxalate. In a metastable supersaturated calcium oxalate solution, a wide molecular weight range of urinary components [2, 11], nephrocalcin [9, 24], and crude extracts of glycosaminoglycans [3] were found to inhibit growth and aggregation of seed crystals of the salt. In artificial urine, at calcium oxalate supersaturation levels that spontaneously precipitated calcium oxalate, Tamm-Horsfall glycoprotein at concentrations of $<10^{-6}$ M and ionic strengths ≤ 0.16, acted as a weak inhibitor of crystal aggregation, but at higher ionic strengths and concentrations it acted as a promoter of crystal aggregation [19]. These activities were observed when the candidate molecules were dissolved in the crystallizing system.

Crystallization-promoting activity was attributed also to Tamm-Horsfall glycoprotein [17] and an unidentified urinary mucoprotein [15]. These were observed respectively in a concentrated urinary environment from which calcium oxalate spontaneously crystallized and under situations which precipitated the macromolecules. That proteins adsorbed on a surface can promote mineral phase nucleation [4] suggests that the crystallization-promoting activities observed earlier could have been mediated by immobilization of the urinary macromolecules concerned.

Studies on the concomitant nucleation-promoting and growth-inhibiting effects of freezing on crystallization of calcium oxalate in whole urine were prompted as a possible means to demonstrate the actions of both solubilized and immobilized macromolecules in the crystallizing system [20]. To mimic the concentrated urinary environment in which stone initiation is considered to occur, we adopted the rapid evaporation method [8] to concentrate test urine to standard osmolality before applying the freezing procedure. In this paper, we report our observations on the characteristics of calcium oxalate crystals that result from freezing ultrafiltrate and retentate fractions of concentrated whole urine of stone-formers and normal controls as further evidence for the dual action of urinary polyanions on the crystallization of calcium oxalate.

Materials and methods

Samples of early morning urine were collected from healthy controls (aged 22–36 years) as well as from stone-formers (aged 25–62 years), transported at 4°C and processed as soon as they reached the laboratory. All urine samples were obtained from male Chinese subjects who were on normal diets.

Table 1. Population density and size of crystals of calcium oxalate dihydrate in concentrated urine (pH 5.3, 1,250 mosmol/kg).

Sample		Normal controls ($n=35$)		Stone-formers ($n=37$)	
		Population density (10^{-5}/ml)	Crystal size (μm)	Population density (10^{-5}/ml)	Crystal size (μm)
[a] Whole urine		1.2 ± 0.3	10.5 ± 0.7	2.8 ± 0.4	9.3 ± 0.5
[b] Fractionated urine					
5 kDa cutoff	R/W	54.0*	0.3*	37.0*	0.4*
	UF/W	0.2**	1.6*	0.2**	2.1*
10 kDa cutoff	R/W	8.1*	0.6*	16.5*	0.5*
	UF/W	0.3*	1.9**	0.2*	1.8*
20 kDa cutoff	R/W	3.0*	0.9	2.4*	0.7*
	UF/W	1.0	1.5*	0.1*	1.7*

Results for [a] whole urine are expressed as mean ± SEM and [b] urine fractionated by ultrafiltration at nominal cutoffs of 5 kDa, 10 kDa and 20 kDa are shown as ratios of retentate: whole urine (R/W) or ultrafiltrate: whole urine (UF/W). (A ratio of >1 for population density of crystals indicates enhanced nucleation and a ratio of <1 for crystal size indicates growth inhibition).
*, ** Statistically different from the corresponding whole urine at $P<0.001$ and $P<0.020$ respectively

Preparation of ultrafiltrate and retentate fractions

Concentrated urine (pH 5.3, 1,250 mosmol/kg) was prepared as described previously [20]. Briefly, this procedure involved acidification of urine to pH 5.3, centrifugation and microfiltration to remove urinary sediments and cellular debris and then rotaevaporation at 37°C to 1,250 mosmol/kg. After the samples had been established to be crystal-free by microscopic examination, ultrafiltration was performed to yield retentates and ultrafiltrates (2:3 v/v) at nominal cutoffs of 5 kDa, 10 kDa and 20 kDa (YM-5, YM-10 membranes, Amicon Corp Model 8010, Danver, Mass., USA; 13249-12 membrane, Sartorius-Centrisart-I, Göttingen, FRG).

Recovery of polyanions from whole urine

The method of Scott and Newton [18] was adopted with minor modifications. In brief, urine samples were centrifuged to remove sediments and then diluted with 3 vol 0.025 M sodium acetate buffer, pH 5.8 and mixed with 0.1 vol cetylpyridinium chloride (5% in acetate buffer). The mixture was allowed to stand overnight at 4°C. The precipitate was washed twice with distilled water and then dissolved in propan-1-ol/water (2:1, v/v). Four volumes of sodium-acetate-saturated ethanol was added to the propanolic solution to reprecipitate the urinary polyanions as sodium salts. The precipitate was washed twice with ethanol and then dried in a dessicator.

Crystallization studies

Samples of whole urine, retentate and ultrafiltrate with or without added urinary polyanions (all at pH 5.3, 1,250 mosmol/kg) were frozen (−20°C) overnight (16-h) to induce crystallization. They were then thawed at 37°C and the population densities and sizes of envelope crystals of calcium oxalate dihydrate therein were determined with the "Improved Neubauer Chamber" haemocytometer.

Statistical analysis

All statistical analyses were performed using the Mann Whitney pair test.

Results

Crystallization in retentate and ultrafiltrate fractions

Crystals that formed in the retentate and ultrafiltrate fractions of concentrated urine (pH 5.3, 1,250 mosmol/kg) were calcium oxalate dihydrates, in the non-aggregated form (Table 1). The population density and size of crystals in each retentate and ultrafiltrate fraction were expressed as rations to those of the corresponding whole urine to minimize effects due to microions and thus demonstrate the effects due to the macromolecular content of each urine sample. It becomes evident then that for both stone-formers and normal controls, ultrafiltration resulted in enhanced nucleation and limited growth of dihydrate crystals of calcium oxalte in the retentates, but had opposite effects in the ultrafiltrates. Urinary macromolecules that were concentrated in the retentate but depleted from the ultrafiltrate must therefore have mediated the observed effects.

Enhancement of crystal nucleation

In the normal controls, enhanced nucleation was about 3 times as high in retentates at a cutoff of 10 kDa as that at a cutoff of 20 kDa. Crystal nucleation in the ultrafiltrates at a cutoff of 10 kDa was accordingly one-third of that the 20 kDa cutoff. Apparently the 10–20 kDa population that was retained by the 10 kDa filter, but which permeated the 20 kDa filter, included molecules responsible for the enhanced nucleation observed. In contrast, the pronounced enhancement in the retentate of the 5 kDa cutoff was not evident in the ultrafiltrate at the 10 kDa cutoff; the 5–10 kDa population retained by the 5 kDa filter but that permeated through the 10 kDa filter thus could not independently enhance nucleation. The observed pronounced enhancement could, however, be

Table 2. Population density and size of crystals of calcium oxalate dihydrate in concentrated urine (pH 5.3, 1,250 mosmol/kg) ultrafiltrate (UF, 10 kDa cutoff) reconstituted with retentate (R, 10 kDa cutoff) or saline (S) (0.9%) at 4:1 (v/v); urine samples of normal controls were studied

Sample	UF+R		UF+S	
	Population density (10^{-5}/ml)	Crystal size (μM)	Population density (10^{-5}/ml)	Crystal size (μm)
1	83.5	3.0	0.8	16.0
2	10.8	7.0	0.2	20.5
3	33.5	5.0	0.2	12.0
4	7.6	2.5	0.2	14.0
5	28.5	2.0	0.3	13.0
Mean	32.8**	3.9*	0.3	15.1
SEM	13.6	0.9	0.1	1.5

*, ** Statistically different from corresponding UF + S at $P<0.001$ and $P<0.050$ respectively

produced by cooperation with populations >10 kDa retained by the 5 kDa filter.

Among the stone-formers, a similar trend of decreasing enhancement in crystal nucleation was observed in retentates at higher cutoffs. However, crystal nucleation in the ultrafiltrates remained low throughout. It follows that crystal nucleation depended more on cooperative activities of urinary macromolecules than on any individual population in this case.

Inhibition of crystal growth

In both stone-formers and normal controls, crystal size in retentates decreased significantly ($P<0.001$) as the cutoffs decreased or the more macromolecules were retained by the filter. This suggests cooperation among the macromolecules in limiting crystal growth. Relief of growth inhibition in the ultrafiltrates, however, did not follow the corollary of this trend. In the normal controls, the difference ($P<0.02$) between ultrafiltrates at the 5 kDa and 10 kDa cutoffs suggests that crystal growth inhibition due to ions and molecules of <5 kDa was reduced by the introduction of an "inert" population of 5–10 kDa. The difference ($P<0.01$) between ultrafiltrates at the 10 kDa and 20 kDa cutoffs suggests then that molecules of 10–20 kDa provided additional growth inhibitory effects to the 20 kDa ultrafiltrate. In the stone-formers, there was no statistical difference between the size of crystals in the ultrafiltrates at the different cutoffs suggesting that molecules of 5–10 kDa or 10–20 kDa did not exert growth inhibitory effects. It appears, therefore, that the macromolecular population (>20 kDa) that is retainable by all three filters was important in limiting crystal growth; populations of smaller size may cooperate in the action, but are not effective as independent growth inhibitors.

Crystallization in ultrafiltrates with macromolecular additives

Ultrafiltrate as solvent. As the crystallization properties of ultrafiltrates at the 5 kDa and 10 kDa cutoffs differed similarly from those at the 20 kDa cutoff in both stone-formers and normal controls (Table 1), they appear not to be contaminated by macromolecular crystal nucleators and growth inhibitors. The ultrafiltrates of either the 5 kDa or the 10 kDa cutoff can therefore conveniently provide the urinary microionic environment for the study of effects of macromolecular additives on crystallization of urinary calcium oxalate. Our choice of ultrafiltrates (10 kDa cutoff) for these experiments was based on the fact that they can be prepared more quickly.

Urinary polyanionic macromolecules. Comparison of crystallization characteristics of urine reconstituted by mixing ultrafiltrates and retentates (4:1, v/v) with corresponding saline controls (Table 2) provided confirming evidence that the retentates contained macromolecules (at ≥kDa) that were capable of nucleating and/or inhibiting growth of calcium oxalate crystals. Polyanions recovered from urine of stone-formers and normal controls were likewise capable of exerting nucleation-promoting and growth-inhibiting effects (Table 3); the higher population density and smaller size of crystals indicated by urine reconstituted with urinary polyanions of stone-formers suggested that urine of stone-formers contained more powerful crystal nucleators and growth inhibitors than that of normal controls.

Table 3. Population density and size of crystals of calcium oxalate dihydrate in concentrated urine (pH 5.3, 1,250 mosmol/kg) ultrafiltrates (10 kDa cutoff) with urinary polyanions of normal controls or stone-formers as additive

Sample	Normal controls ($n=16$)		Stone-formers ($n=10$)	
	Population density (10^{-5}/ml)	Crystal size (μm)	Population density (10^{-5}/ml)	Crystal size (μm)
Ultrafiltrate	0.3±0.1	17.8±1.2	0.4± 0.2	15.8±2.3
Ultrafiltrate + urinary polyanions	3.1±0.8**	7.8±0.6*	108.3±47.3***	3.0±0.4*

The final concentration of the additive was 4–8 mg/ml. Results are expressed as mean ± SEM
*, **, *** Significantly different from corresponding ultrafiltrate at $P<0.001$, $P<0.002$, and $P<0.050$ respectively

Discussion

In the present study, ultrafiltration was employed as a means of demonstrating the gross effects of urinary macromolecules on crystallization of urinary calcium oxalate in the concentrated microionic environment of urine. Since the concentrations of small ions and molecules in test urine remain essentially unchanged with ultrafiltration [17], any difference in crystallization between retentate, ultrafiltrate and original whole urine would be due to the difference in concentration of macromolecules therein. Urinary macromolecules concentrated in retentate but depleted from ultrafiltrate were thus shown to be active in inducing nucleation and inhibiting growth of calcium oxalate crystals in urine. In a similar dehydrated urinary environment, decrease in calcium oxalate crystal formation in the urine ultrafiltrate was likewise recovered with the addition of uromucoid [17]. It was on the basis of these observations that we decided to employ the urine ultrafiltrate, devoid of crystal-active macromolecules, to provide the metastable microionic environment for the introduction of mineralization agents and study of subsequent effects on crystallization in the urinary environment.

Controversy about the functions of urinary macromolecules appears to be related to the physical state in which they were studied. In a solution which contains only lithogenic ions or in artificial urine of microion composition similar to that of urine, the macromolecular additive, present in low concentrations, occurs as a hydrated moiety free to interact with ions or crystallites. Such conditions have been shown to inhibit calcium oxalate precipitation [2], inhibit crystal aggregation [10] and inhibit crystal growth [6]. At higher concentrations of macromolecules or in complex solutions containing a heterogeneous population of macromolecules, intermolecular interaction results in polymerization and aggregation to various sizes that can or cannot remain in solution. Such conditions have indeed been found to promote calcium oxalate crystal aggregation [19], promote precipitation of calcium oxalate crystals [8], and promote nucleation but inhibit growth of calcium oxalate crystals [4, 6, 21]. The concomitant nucleation-promoting and growth-inhibiting effects that we observed with freezing [20] were thus also observed with polyelectrolytes and proteins in solution as well as immobilized on a microscope slide [4, 6] or in a gel [12, 21]. The frozen state served to immobilize polyanions, some of which provide charged groups that are correctly oriented to stabilize the ionic nucleus of calcium oxalate and allow the crystal lattice to grow in space. Others that remain mobile adsorb on the active crystal sites to arrest growth, which is a situation analogous to the matrix in biomineralization [1]. In the present study, the concentration of the macromolecules in the retentate further promoted intermolecular interaction, which possibly facilitated the observed influence on crystal nucleation and inhibition of crystal growth. Alternatively, increased crystal nucleation and limited crystal growth in the presence of macromolecules have been suggested to be secondary to inhibition of aggregation by macromolecules [7].

We have previously reported [20], using a similar test system, that urine samples of stone-formers crystallized calcium oxalate at higher population densities and smaller sizes than those of normal controls, an effect which may be mediated by urinary polyanions. Supporting evidence was provided in the present study, in which consideration of the ultrafiltrate fraction of urine revealed effects due to separate macromolecular populations. Our attention was therefore focused on urinary macromolecules in the nominal size range of 5–20 kDa. Of these, the 10–20 kDa population in normal controls behaved as promoters of nucleation and inhibitors of growth, whereas in the size range studied no particular population in the stone-formers behaved similarly. Consideration of crystallization inhibitory activity in diluted ultrafiltrate fractions of urine revealed similar differences in the active size range of urinary macromolecules between normal controls and stone-formers [2]. As an alternative means of size fractionation, gel filtration of urine from stone-formers and normal controls yielded fractions in the range 17–>60 kDa that could inhibit growth and/or promote nucleation of crystals of calcium oxalate monohydrate to various degrees [11]. Therefore, urinary macromolecular modifiers of calcium oxalate crystallization occur over a wide range of molecular weights which include both glycoproteins and glycosaminoglycans. Further attempts at a clear size fractionation will inevitably encounter the difficulty of analyzing overlapping populations of heterodisperse hydrodynamic sizes that are characteristic of such polyanionic macromolecules as the glycosaminoglycans [22]. It is important to note that the hydrodynamic sizes of glycosaminoglycans dictate that they behave on gel filtration as though they were much larger molecules than would be expected from proteins of comparable molecular weights [22], thus necessitating the differentiation between proteins and polyanionic glycosaminoglycans in any analysis of molecular size.

In the present study, consideration of the retentate fractions provided additional information about the cooperative effects of urinary molecules which independently did not show apparent effects. In normal controls, the 5–10 kDa population was apparently inert, but cooperated with the crystal-active 10–20 kDa population in producing pronounced enhancement of crystal nucleation. In stone-formers, no particular population of urinary macromolecules in the 5–20 kDa range was capable of independently promoting nucleation and inhibiting growth, though they appeared cooperatively to exert pronounced effects. Cooperation may have been brought about by macromolecular interaction which, in turn, was facilitated by being concentrated in the retentate.

Polyanions recovered from urine of stone-formers were shown in the present study to be more powerful than those from normal controls as promoters of nucleation and inhibitors of growth of calcium oxalate crystals from the concentrated urinary environment. The polyanions so recovered have been reported to include chondroitin-dermatan sulphate, heparan sulphate and glycoproteins [18]. The glycosaminoglycan components in the polyanionic extract were estimated to fall in the range of 8–30 kDa

[23], whereas such glycoproteins identified as nephrocalcin [13] and Tamm-Horsfall [16] were found to be 14 kDa and 85 kDa respectively in the monomeric state. Therefore, glycosaminoglycans fall within the crystal-active fractions observed in the present study. While no significant difference in the 24-h average excretion of glycosaminoglycans between normal and stone-forming subjects was found [5], further investigation into possible structural differences between glycosaminoglycans excreted by the two groups is limited by the low concentrations in urine and the need for non-destructive separation from co-extracted glycoproteins. Reported actions of the glycosaminoglycans on crystallization of calcium oxalate range from negligible [10], inhibitory [3] to promoting [14]. The actions of urinary glycosaminoglycans have yet be studied as co-existing solubilized and immobilized components in a concentrated urinary microionic environment to appreciate their influence on crystallization of urinary calcium oxalate.

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