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Urinary macromolecular inhibition of crystal adhesion to renal epithelial cells is impaired in male stone formers

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Background. Retention of microcrystals that form in tubular fluid could be a critical event in kidney stone formation. This study was performed to determine if urinary macromolecules from stone-forming (SF) individuals have reduced ability to inhibit crystal adhesion to renal cells.

Methods. A first morning whole urine (WU) sample was obtained from 24 SF subjects (17 males and 7 females) and 24 age-, race-, and sex-matched controls (C). An aliquot of urine was centrifuged and an ultrafiltrate (UF) free of macromolecules >10 kD and 10× concentrate (U_{conc}) were prepared.

Results. Supplementing UF with increasing amounts of U_{conc} to return the macromolecule concentration to 0.25×, 0.5×, or 1× of baseline progressively decreased crystal binding to cells. This effect was blunted in the male SF group compared to controls ($P < 0.05$, SF vs. C, for UF plus 0.25× macromolecules). No difference was apparent in the female groups. In order to identify responsible macromolecule(s), calcium oxalate monohydrate (COM) crystals were coated with U_{conc} and adherent proteins then released and probed by Western blot. Coated COM crystals from male controls contained 3.5-fold more Tamm-Horsfall protein (THP) than SF subjects ($P < 0.01$). COM crystal coating with other proteins did not consistently differ between the groups. COM crystal coating by urinary prothrombin fragment 1 (UPTF1, $P < 0.05$) and crystal adhesion inhibitor (CAI) ($P = 0.09$) correlated with decreased crystal binding to cells, whereas coating with osteopontin (OPN) correlated with increased adhesion tendency ($P < 0.05$).

Conclusion. Urinary macromolecules >10 kD coat COM crystals and block their adhesion to renal cells. This capacity appears to be blunted in male but not female SF individuals. Multiple urinary proteins may play a role in renal cell-urinary crystal interactions, and THP appears to be one of the more important ones.

Key words: MDCKI cells, nephrolithiasis, osteopontin, Tamm-Horsfall protein, urinary prothrombin fragment 1.

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Urine is often supersaturated so that the formation of calcium oxalate or calcium phosphate crystals is favored [1]. However, unless these small crystals grow large enough to occlude a tubule lumen, aggregate with other crystals to form a mass large enough to do so, or adhere to the tubular epithelium, any intraluminal crystals will be swept out of the nephron in the flowing fluid within a few minutes [2, 3]. In many calcium oxalate stone formers, medullary interstitial calcium phosphate deposits appear to precede the formation of larger stones [4], although the processes that mediate interstitial crystal deposition and evolution into calcium oxalate stones remain to be defined, and it is conceivable even these interstitial crystals originate within tubular lumen. In more marked hyperoxaluric states (e.g., enteric or primary hyperoxaluria) the pathology differs, and direct adhesion of calcium phosphate or oxalate crystals to renal epithelial cells, or initial damage of tubular epithelium by crystals or oxalate ion, may be important [4]. Therefore, we and others have studied the response of renal tubular cells to oxalate ions and calcium oxalate and calcium phosphate crystals [5–13], since these events could trigger a series of events that lead to pathologic renal calcification.

Adhesion of calcium oxalate crystals to anionic molecules on the surface of renal epithelial cells is crystal-face specific [14] and can be blocked by competing soluble anions in tubular fluid, such as glycosaminoglycans, citrate, or glycoproteins [5, 6]. Whole urine itself can block crystal adhesion to cells [15], and osteopontin (OPN) [6], nephrocalcin [6], heparan sulfate [6], bikunin [16], and the recently described crystal adhesion inhibitor (CAI) [17] have each been found active when tested in isolation. It has been postulated that crystallization inhibitors in the urine of certain stone formers might be defective, thereby explaining their disease [1]. Studies on crystallization in the presence of whole urine and fractions isolated from it have lent support to this hypothesis [18–22]. Therefore, in this study we tested the hypothesis that whole urine from stone-forming individuals is defective containing macromolecules that coat crystals

Table 1. Baseline parameters of stone-forming patients

			Serum		24-hour urine								Urinary supersaturation				
	Age	# Stones	Cr	Ca	pH	Vol	Cit	Ox	Ca	Ph	UA	Mg	CaOx	Br	Ap	UA	NaUr
Male mean (17)	56.7	2.6	1.2	9.7	6.0	2042	709	0.36	297	1175	836	144	2.04	0.25	3.78	2.45	2.12
Male SD	12.5	1.3	0.1	0.2	0.3	897	300	0.09	114	355	146	46	0.35	1.01	1.65	1.93	0.74
Female mean (7)	54.0	4.9	1.1	9.5	6.0	1680	537	0.35	215	862	610	100	1.80	0.13	3.41	0.78	1.32
Female SD	12.8	7.6	0.2	0.3	0.5	821	266	0.13	96	434	198	37	0.30	1.02	1.75	2.95	1.00
All mean	55.4	3.8	1.1	9.6	6.0	1856	623	0.35	249	997	706	120	1.91	0.24	3.70	1.55	1.71
All SD	13.0	5.7	0.2	0.3	0.4	898	303	0.11	112	436	206	48	0.35	1.04	1.73	2.52	0.95

Abbreviations and units are: serum, creatinine (Cr, mg/dL) and calcium (Ca, mg/dL); urine volume (vol, mL), citrate (Cit, mg), oxalate (Ox, mmol/L), calcium (Ca, mg), phosphorous (Ph, mg), uric acid (UA, mg), and magnesium (Mg, mg). Urinary supersaturation in D.G. units for calcium oxalate [CaOx, brushite (Br), apatite (Ap), uric acid (UA), and sodium urate (NaUr)].

less effectively, thereby favoring crystal retention in the kidney.

METHODS

Patient population

Patients with idiopathic calcium oxalate stone disease, confirmed (analysis of passed stone) or suspected (radiopaque by radiographic imaging in the absence of known secondary causes such as gastrointestinal disease or renal tubular acidosis), were recruited from the Mayo Stone Clinic (17 men and 7 women). Controls were carefully matched for age (within 5 years), sex, and race, and lacked a history of renal stone disease. Baseline characteristics of the stone formers before treatment are seen in Table 1. As a group, compared to females, male stone formers had passed less stones (2.6 vs. 4.9), had a higher urine volume (2042 vs. 1679 mL), and had a tendency toward hypercalciuria (297 vs. 215 mg/day) and hyperuricosuria (835 vs. 610 mg/day). As expected, given the criteria of the study, calcium oxalate supersaturation was increased for both groups (2.04 and 1.80 D.G., respectively, with an upper limit of normal of 1.75 D.G.). For men and women the mean time since the first stone event was 14 years and 4 years, and most recent stone event 3 years and 2 years, respectively. Patients were maintained on their standard stone treatment protocol for this trial (thiazide in 7, potassium citrate in 6, and allopurinol in 2).

Cell culture

Renal epithelial cells of the Madin-Darby Canine Kidney (MDCK) line, type I, were a generous gift of Carl Verkoelen (Erasmus University, Rotterdam, The Netherlands). Cells were grown in Dulbecco-Vogt modified Eagle's medium containing 25 mmol/L glucose (DMEM) at 38°C in a CO_2 incubator as previously described [13]. To prepare high-density, quiescent cultures, 1×10^6 cells/35-mm plastic plate (9.62 cm^2; Nunc, Naperville, IL, USA) were plated in DMEM containing 10% calf serum and 1.6 μmol/L biotin. Two days later, when they were confluent, the medium was aspirated and replaced with fresh medium containing 5% calf serum and 1.6 μmol/L biotin. The established monolayer was used for study the next day.

Preparation of human urine samples

A first morning urine sample was obtained from each subject and control. Whole urine (WU) was centrifuged ($200g \times 10$ min) to remove cells and debris. Urine osmolarity was assessed using an osmometer, and 100 mL of urine was concentrated ~10-fold using an Amicon stir cell fitted with a 10-kD cutoff membrane to produce ultrafiltrate (UF) and 10 mL of 10×-concentrate (C). The starting volume of urine that was reduced to 10 mL, and hence, the degree of concentration, was normalized between samples (e.g., if the U_{osm} was 650 mOsm/kg then 100 mL of urine was concentrated, if 325 mOsm/kg then 200 mL was employed, and if 1300 mOsm/kg then 50 mL was employed). In this manner, the same quantity of macromolecules, namely those in 100 mL of urine with a U_{osm} 650 mOsm/kg, was contained in each final 10 mL concentrate. Total 24-hour urinary osmol excretion is proportionate to body size, and has, therefore, been used to normalize excretion of urinary analytes between patients, similar to creatinine [23]. A U_{osm} of 650 mOsm/kg was chosen as the target in order to mimic a typical milieu of the distal tubule under conditions of modest dehydration, while standardizing between patients. The average first morning U_{osm} did not differ significantly between patients and controls as a group. Artificial urine (AU) was prepared as previously described [24].

Adhesion of crystals to cells

To measure adhesion of crystals to cells, the culture medium was aspirated and the cells rinsed once with phosphate-buffered saline (PBS, pH 7.4) and covered with 2 mL of WU or UF. To the selected plates containing UF, urine concentrate (C) was added (0.2 mL) to bring the concentration of macromolecules back to their ambient urinary concentration (1×). [^{14}C] calcium oxalate monohydrate (COM) crystals were then added to the cells, achieving a final concentration of 200 μg/mL (41.6 μg/cm^2 of cells). The culture dishes were gently