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Dawn Durrbaum · Allen L. Rodgers
Edward D. Sturrock

A study of crystal matrix extract and urinary prothrombin fragment 1 from a stone-prone and stone-free population

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Abstract South African blacks are immune to urinary calculi whereas whites have an incidence rate similar to that reported in Western societies. Urinary prothrombin fragment 1 (UPTF1) and the crystal matrix extract (CME) from which it is derived have been shown to be potent inhibitors of crystal growth and aggregation in undiluted human urine. The objective of the present study was to isolate CME and UPTF1 from the urines of black and white subjects in order to assess whether either might contribute to the black population's relative stone immunity. CME was isolated from freshly precipitated calcium oxalate (CaOx) crystals and a crystallization study was conducted in synthetic urine. Coulter Counter, ^{14}C-oxalate deposition, and scanning electron microscopy data demonstrated that the extracts from both race groups strongly inhibited CaOx nucleation. The extract derived from the black subjects inhibited nucleation to a greater extent than that from the whites. A phase conversion from COM to COD in the presence of the extracts, in support of the inhibitory effect of CME, was also observed. Purified UPTF1 isolated from both groups' CME was subjected to rigorous biochemical characterization involving matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, protein sequencing by Edman degradation, and amino acid analyses. No differences in molecular weight or amino acid sequence and composition were identified. It is suggested that the more potent inhibitory activity of the extract derived from the black subjects might be related to this group's relative stone immunity.

D. Durrbaum (✉) · A. L. Rodgers
Department of Chemistry, University of Cape Town, South Africa
e-mail: dawn@psipsy.uct.ac.za

E. D. Sturrock
Department of Medical Biochemistry, University of Cape Town, South Africa

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Introduction

South African blacks are immune to urinary calculi while whites have an incidence rate similar to that reported in Western society [20]. The recently reported prevalence of nephrolithiasis in blacks of less than 1% [13] confirms earlier observations of the rarity of renal stones in this race group relative to whites [14].

Despite this apparent stone immunity, urinalysis data in blacks show trends contrary to those which might have been expected. For example, Meyers has shown lower urinary calcium and citrate excretion in black subjects [13] while studies in our laboratory have demonstrated higher oxalate and lower citrate levels in blacks relative to whites [11].

Although urine composition has received much attention, an aspect which has not yet been investigated in blacks is the role of urinary proteins such as Tamm-Horsfall glycoprotein [9], nephrocalcin [4], uropontin [17], and inter-α-inhibitor [1, 5, 12], which have been shown in contemporary studies to have inhibitory properties. Of particular interest and promise is urinary prothrombin fragment 1 (UPTF1), one of very few proteins whose inhibitory potential has been tested in urine and which therefore may have some implications on actual stone formation. This 31 kDa glycoprotein has been shown to be a potent inhibitor of calcium oxalate crystal growth and aggregation in undiluted human urine [15]. Furthermore, a crude precursor to the purified protein has been shown to inhibit the crystallization process potently [6].

The objective of the present study was therefore to isolate and purify UPTF1 from the urine of South African black and white subjects, with a view to comparing their inhibitory activity and biochemical

properties, and thereby assess whether UPTF1 might be a contributory factor regarding the former group's apparent stone immunity.

Materials and methods

Subjects and urine treatment

Twenty-four-hour urine samples were collected in plastic bottles containing a boric acid preservative to prevent calcium oxalate precipitation (the absence of crystals was confirmed by light microscopy). The subjects were healthy urban white and rural black males on their free unrestricted diet with no previous history of kidney stones. Urines testing positive for haematuria or infection using urinalysis test strips were excluded. A maximum of 10 urines were pooled according to race and filtered through a 0.75 μm pre-filter and 0.45 μm nitrocellulose filter paper before use.

Isolation of crystal matrix extract

Crystal matrix extract (CME) was isolated from pooled urines according to the method of Ryall et al. [15]. In this protocol, calcium oxalate (CaOx) crystals were freshly precipitated from filtered urine by the addition of sodium oxalate in 30 mM excess of the urinary metastable limit, while incubating at 37 °C in a shaking water bath for 4 h. These crystals were then filtered through 0.22 μm Millipore filters, washed and demineralized in 0.25 M EDTA (pH 8.0), yielding the CME.

Inhibitory activity of CME

Three replicate crystallization studies were conducted independently to assess and compare the effect of the crystal matrix extracts from both population groups on CaOx crystallization in a synthetic urine, pH 6.2 (SU) [2]. After measuring the metastable limit of the synthetic urine, it was divided into three flasks that were incubated at 37 °C in a shaking water bath. Equal volumes of distilled water or 5 mg/l of CME derived from whites (WE) and blacks (BE) were added to these flasks, henceforth referred to as the control, WE-dosed and BE-dosed urines, respectively. A 10 mM dose of sodium oxalate in excess of the synthetic urine's metastable limit was then added to each flask, and the crystallization rates in terms of particle numbers and particle volume were monitored for 1 h using a Coulter Counter. The CaOx supersaturation of the synthetic urine samples before and after addition of sodium oxalate were both computed using the programme EQUIL and were 3.12 and 6.39, respectively [19]. Aliquots of crystals were retained for scanning electron microscopy after the first experiment only.

Since it has been reported that macromolecules trapped within crystalline architecture as well as crystal interstices can erroneously contribute to particle volume measured by a Coulter Counter [6, 15], crystal deposition by ^{14}C-oxalate was measured at 30 min in parallel with Coulter Counter analyses. Experiments were carried out as described above, except that 3.125 μCi/100 ml "hot" ^{14}C-oxalate was added to each flask prior to addition of the "cold" sodium oxalate. Samples were filtered into concentrated hydrochloric acid (10% v/v) of which duplicate 1 ml aliquots were added to 10 ml scintillation fluid (Zinsser Analytic, Frankfurt, Germany) and each sample was counted for 10 min in a scintillation counter.

Purification of UPTF1

The CMEs obtained from both population groups were dialysed extensively against distilled water at 4 °C using a membrane with a 10 kDa cut-off, freeze-dried, and desalted on a P2 BioGel column using a 25 mM Tris-HCl buffer (pH 7.3). Finally, UPTF1 was isolated from the desalted protein mixture by RP-HPLC with a Vydac C4 column. A 50 min linear gradient was developed from 0 to 60% B, where solvent A was 0.1% trifluoroacetic acid (TFA) and solvent B was 0.1% TFA in 100% acetonitrile. The UPTF1 peaks identified by SDS-PAGE were pooled and dialysed against a 10 mM phosphate buffer (pH 7.3). The protein concentration of the dialysed material was determined using a BioRad protein assay. SDS-PAGE followed by silver staining was used to monitor the isolation procedure and to demonstrate the purity of the protein collected after HPLC.

Characterisation of UPTF1

Proteins were resolved by SDS-PAGE on 15% polyacrylamide gels and analysed by Western blotting using a monoclonal UPTF1 antibody (provided by Professor Rosemary Ryall, Flinders Medical Centre, South Australia) at a dilution of 1:500. Purified UPTF1 from black and white subjects was resolved and protected with vinyl pyridine, followed by hydrolysis with endoproteinase Lys-C (2%, w/w). The total digest was analysed directly by MALDI-TOF mass spectrometry, as described elsewhere [16]. Mass spectra were usually generated from a sinapinic acid matrix (10 mg/ml) using a Perceptive Voyager Elite Biospectrometry Workstation. Amino acid analyses and protein sequencing by Edman degradation were also carried out on aliquots of purified UPTF1 from both black and white population groups.

Results

Isolation of CME and UPTF1

Pooled urine in 15–20 litre batches yielded 4–5 g and 7–9 g of CaOx crystals (confirmed by X-ray powder diffraction) from the blacks and whites, respectively, and 300–500 μg purified UPTF1. The isolation process was monitored at various stages using SDS-PAGE followed by silver staining and is depicted in Fig. 1a.

A prominent band with a similar relative mobility to that of UPTF1 appeared in most urine fractions (prominent in BF and BS). Western blotting demonstrated that this band was not UPTF1. Its identity was not investigated further in this study.

The crystal matrix extracts (lane 4: BE and lane 8: WE) both showed a dominant band with an apparent molecular weight of 40 kDa, which was identified as UPTF1; however, other bands were also visible. Although not identified in this study, the additional bands could be due to other proteins such as osteopontin, albumin, inter-α-inhibitor, and microglobulins. This confirmed previously reported observations that UPTF1 is the most abundant protein incorporated into CaOx crystals [7]. The UPTF1 appeared to be more prevalent in the crystals derived from black pooled urine than their white counterparts, although Western blotting (Fig. 1b) did not confirm this (refer to lanes 1 and 3: WE and BE). It is noted that the second-most abundant protein in the crystals from the black pooled urine had a molecular weight less than UPTF1 (the identity of which is unknown), while the second-most abundant protein from the white pooled urine had a higher molecular weight than UPTF1 (an approximate weight of 67 kDa suggests it could correspond to albumin).

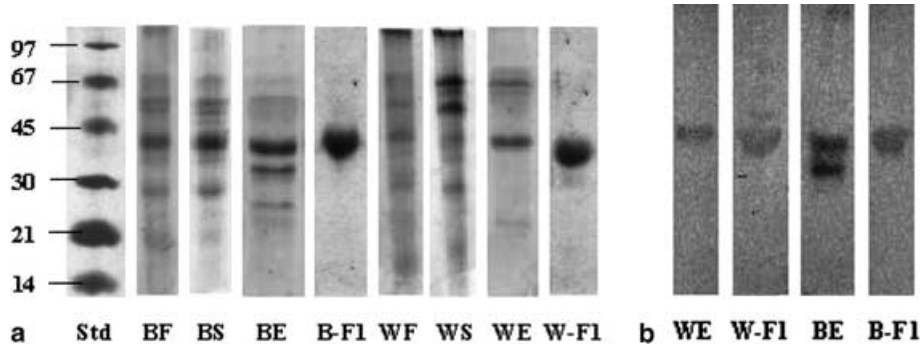

Fig. 1 SDS-PAGE of matrix proteins extracted from CaOx crystals. **a** SDS-PAGE. Silver stained SDS gel: *lane 1* (*Std*): low-molecular-weight standards, *lane 2* (*BF*): filtered urine from black males, *lane 3* (*BS*): supernatant urine from black males, *lane 4* (*BE*): crystal matrix extract from black males, *lane 5* (*B-F1*): purified UPTF1 from black males, *lane 6* (*WF*): filtered urine from white males, *lane 7* (*WS*): supernatant urine from white males, *lane 8* (*WE*): crystal matrix extract from white males, *lane 9* (*W-F1*): purified UPTF1 from white males. **b** Western blot. The patterns were obtained from: *lane 1* (*BE*): crystal matrix extract from black males, *lane 2* (*B-F1*): purified UPTF1 from black males, *lane 3* (*WE*): crystal matrix extract from white males, *lane 4* (*W-F1*): purified UPTF1 from white males

The protein obtained after RP-HPLC consisted of a single protein band and was therefore regarded as sufficiently pure for amino acid, molecular weight, and sequencing determinations (lane 5: B-F1 and lane 9: W-F1).

Inhibitory activity of CME

Effect of CME on particle number and volume

Since CME from both race groups did not alter the metastable limit of synthetic urine, equal amounts of sodium oxalate were added to the control and extract-dosed samples in three independent sets of experiments. Figure 2 shows the increase in particle number above 2.8 μm during a 1-h time course after addition of an oxalate load. The particle number trends of the extract-dosed urines relative to the control were demonstrated in three experiments. Two features were apparent: firstly, CME from both race groups caused a significant decrease in particle number relative to the control and secondly, the extent of the decrease was significantly greater in the urine dosed with BE. The mean particle numbers after 30 min were 23.106, 17.644 and 8.408 in the SU and WE and BE-dosed urines, corresponding to 23.6% and 63.6% inhibition in the latter two urines, respectively. These values correspond to the point of maximum inhibition during the time period investigated. Figure 3 shows the particle volume during the same time course and indicates a decrease in particle volume in the presence of both CMEs. The BE-dosed urine showed a greater decrease in volume (corresponding to 37.6% inhibition) relative to the control than the WE-dosed urine (corresponding to 29.2% inhibition) with mean particle volumes of 2.333, 2.118 and 1.674×10^6 μm^3/500 μl in SU, WE and BE, respectively (at 45 min). These effects were observed in two of the three experiments.

Effect of CME on CaOx deposition

The mean amount of ^{14}C-oxalate remaining in solution was measured after 30 min and is presented as a percentage of the zero time value. The mean amounts remaining were 80.9% (±1.1%) and 91.6% (±0.2%) in the WE and BE-dosed urines compared to 78.1% (±1.1%) in the untreated urine, corresponding to inhibition of CaOx deposition of 3.6 and 17.3%, respectively, in the dosed urines. These data demonstrate that

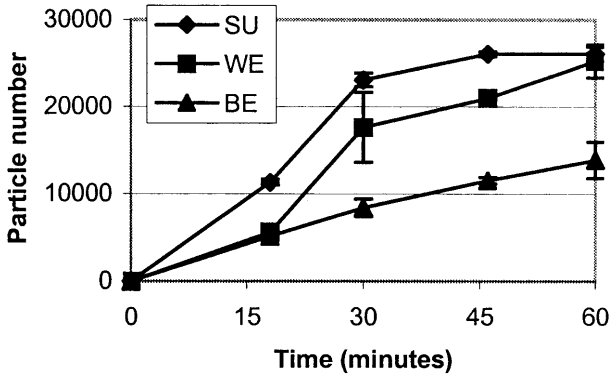

Fig. 2 Effect of 5 mg/l crystal matrix extracts derived from white (*WE*) and black (*BE*) subjects on particle number after addition of an oxalate load to synthetic urine (*SU*)

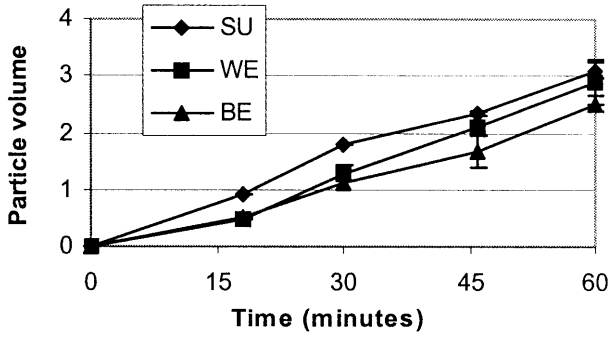

Fig. 3 Effect of 5 mg/l crystal matrix extracts derived from white (*WE*) and black (*BE*) subjects on particle volume* after addition of an oxalate load to synthetic urine (*SU*)

the amount of CaOx deposited in the synthetic urine decreased in the presence of both CMEs and that BE inhibited CaOx deposition to a greater extent than WE.

Effect of CME on particle size

The average particle diameter precipitated from the untreated SU and the extract-dosed samples was measured by the Coulter Counter after 60 min. Neither extract significantly altered the particle size relative to the untreated synthetic urine. The average particle size was 4.0 μm (data not shown). These results were confirmed by scanning electron microscopy, which showed mainly single CaOx crystals with cross-sectional diameter ≤5 μm in the SU and extract-dosed urines.

Figure 4 shows scanning electron micrographs of crystals precipitated from the synthetic urine in the absence (SU) and presence of the extracts derived from white (WE) and black (BE) subjects. In the untreated SU, aggregated (Fig. 4a) and single calcium oxalate monohydrate crystals (COMs) were typically observed. It is noteworthy that on the evidence of scanning electron micrographs, addition of the CMEs resulted in the formation of calcium oxalate dihydrate crystals (CODs), either by direct formation or transformation of COMs to CODs. Single CODs together with a few COD aggregates were observed in both extract-treated SUs. An example of the aggregates is shown in Fig. 4b. Furthermore, survey views of the three samples illustrate a marked decrease in the number of single and aggregated crystals in the presence of both extracts (Fig. 4d, e) compared to the untreated SU (Fig. 4c).

Characterization of UPTF1

In order to investigate possible differences between the protein backbones, MALDI-TOF, amino acid analysis, and protein sequencing by Edman degradation were used. Denatured, reduced, and vinyl pyridine-protected UPTF1 was digested with endoproteinase Lys-C and Asp-N that cleave peptide bonds C- and N-terminally at lysine and aspartic acid respectively. Whole or HPLC fractionated digests were analysed by MALDI-TOF mass spectrometry (Table 1). The spectra revealed, inter alia, $[M+H]^+$ ions at m/z of 1207.7, 766.20, and 1618.3 which correspond closely to the calculated masses of the Lys-C derived peptides Ala-1–Lys-10, Asp-38–Lys-43, and Tyr-44–Lys-56. Ions at 2000.36 and 1118.56 corresponded to Asp-N derived peptides Asp-130–Gln-145 and Asp-146–Arg-155. Thus, the mass spectral data are consistent with the reported sequence of UPTF1.

An amino acid analysis and a five amino acid N-terminal protein sequence by Edman degradation were in agreement with Walz [18] (data not shown) and thus confirmed the identity of the purified protein as UPTF1.

These analyses did not indicate any quantitative differences between blacks and whites.

Discussion

The observation in the present study that particle numbers, volume, and total ^{14}C-oxalate deposition decreased in SU after addition of WE and BE, indicates that the crystal matrix extract, irrespective of the race group from which it has been derived, is an inhibitor of CaOx nucleation in synthetic urine. Quantitative particle size data from the Coulter Counter and semi-quantitative scanning electron microscopy support this mechanism rather than one involving a promotion of aggregation, which may have been a possible interpretation of Coulter Counter data alone. Inhibition of nucleation by CME has not been demonstrated previously by other workers.

In a study by Doyle [6], crystal matrix extract was shown to be an inhibitor of CaOx growth and aggregation rather than nucleation. However, that study was conducted in a seeded crystallization system and undiluted ultrafiltered human urine and therefore differed substantially with our protocol. Doyle postulated that the observed inhibitory properties were largely attributable to the extracts' major component, UPTF1. The synthetic urine employed in the present study contains several more components than Doyle's seeded system but far less than the complex milieu of real urine itself. The disparate complexity of these three test solutions is a likely explanation for the different mechanisms of inhibition exhibited by the crystal matrix extracts. Nevertheless, the synthetic control chosen for the present study satisfied the requirement of a neutral test solution in which to compare protein extracts derived from the two population groups since the use of a real urine from either group may have influenced the relative effects. The important point which emerges, however, is that CME appears to have the capacity to inhibit all three crystallization mechanisms, but that its particular inhibitory role is dependent on the nature of the chemical milieu of the test solution.

A noteworthy observation of the present study was the phase conversion from COM to COD in the presence of the extracts. Such a phase conversion is consistent with the inhibitory properties demonstrated by both CMEs since Cerini showed that COMs are usually found in stone formers' urines but seldom in healthy urine [3]. According to Cerini, the CODs' higher positive charge leads to repulsive forces between crystals and thus disaggregation, while their lower negative charge diminishes crystal adhesion to the cell surface, thereby reducing the retention of crystals in the collecting ducts. Thus, the formation of COD brought about in the presence of CME may be regarded as a favourable primary step, ultimately leading to secondary inhibitory processes. The aforementioned phase conversion is consistent with the observation of Fleming that intra-

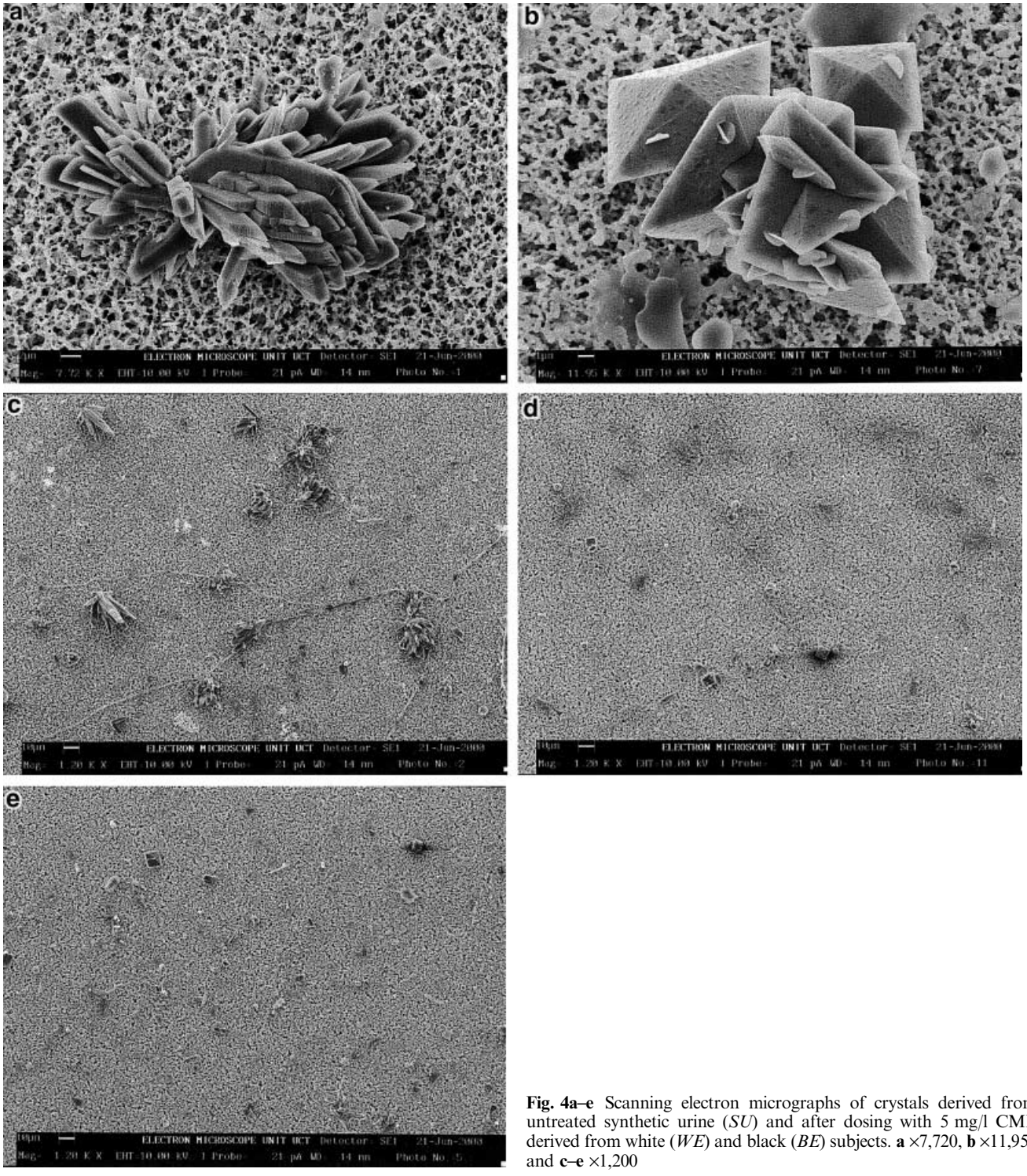

Fig. 4a–e Scanning electron micrographs of crystals derived from untreated synthetic urine (*SU*) and after dosing with 5 mg/l CME derived from white (*WE*) and black (*BE*) subjects. **a** ×7,720, **b** ×11,950 and **c–e** ×1,200

crystalline proteins may play directive roles in CaOx crystal formation [8].

Of interest is our observation that the extract derived from black subjects inhibited crystal nucleation to a significantly greater extent than that from white subjects. Indeed, the inhibition of particle numbers by BE was 40% more than WE. This is suggestive of a possible superior inhibitory capability that may be related to the black population group's relative stone immunity.

Recognizing that UPTF1 is the major component of the extracts, it is tempting to speculate that the observed inhibitory effects are due to the former. While this seems likely in light of Ryall's UPTF1 study [15], several other proteins as well as non-proteinaceous material such as lipids could contribute to the extracts' inhibitory activ-

Table 1 MALDI-TOF of observed $[M + H]^+$ ions of UPTF1 generated by endoproteinase Lys-C or Asp-N (cysteines were protected with vinyl pyridine)

a.a. residues	Calculated m/z	Observed m/z
1–10	1207.35	1207.7
38–43	765.88	766.20
44–54	1374.56	1375.46
44–56	1617.8	1618.3
130–145	1999.32	2000.36
146–155	1118.29	1118.56

ity. However, even if CME's activity could be directly attributed to UPTF1, it is cautioned that these results cannot be readily extrapolated to activity in real urine or even to a physiological function in actual stone formation. Nevertheless, the present study provides sufficient evidence to suggest that an investigation of UPTF1, rather than the crude crystal matrix extracts tested here, from blacks and whites in *real* urine is warranted. Comprehensive biochemical characterization of UPTF1 showed no significant differences in amino acid composition, protein sequence, MALDI-TOF peptide fragments, or pI values (data not shown) in blacks and whites. This suggests that the enhanced inhibition demonstrated by the blacks' CME cannot be explained by structural differences in the backbone of the UPTF1 molecule. Further studies of UPTF1 glycosylation patterns from black and white subjects may explain the differences in inhibitory activities observed [10, 21]. Such a glycosylation study, in conjunction with crystallization studies of UPTF1 in real urine should therefore clarify the contribution, if any, of this inhibitor to the stone immunity of South Africa's black population.

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References

1. Atmani F, Mizon J, Khan SR (1996) Inter-alpha-inhibitor: a protein family involved in the inhibition of calcium oxalate crystallisation. Scan Micros 10: 425
2. Burns JR, Finlayson B (1980) A proposal for a standard reference artificial urine in in vitro urolithiasis experiments. Invest Urol (Berl) 18: 167
3. Cerini C, Geider S, Dussol B, Hennequin C, Daudon M, Veesler S, Nitsche S, Biostelle R, Berthézne P, Dupuy P, Vazi A, Berland Y, Dagorn J-C, Verdier J-M (1999) Nucleation of calcium oxalate crystals by albumin: Involvement in the prevention of stone formation. Kidney Int 55: 1776
4. Coe FL, Parks JH, Nakagawa Y (1991) Protein inhibitors of crystallisation. Semin Nephrol 11: 98
5. Dawson CJ, Grover PK, Ryall RL (1998) Inter-α-inhibitor in urine and calcium oxalate urinary crystals. Br J Urol 81: 20
6. Doyle IR, Marshall VR, Dawson CJ, Ryall RL (1995) Calcium oxalate crystal matrix effect: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in undiluted human urine in vitro. Urol Res 23: 53–62
7. Doyle IR, Ryall RL, Marshall VR (1991) Inclusion of proteins into calcium oxalate crystals precipitated from human urine: a highly selective phenomenon. Clin Chem 37: 1589
8. Fleming DE, Doyle IR, Evans N, Marshall VR, Parkinson GM, Ryall RL (1999) Proteins associated with calcium oxalate crystals formed in human urine are intracrystalline. In: Borghi L, Meschi T, Briganti A, Schianchi T, Novarini A (eds) Proceedings of the 8th European Symposium of Urolithiasis, Editoriale Bios, Cosenza, p 359
9. Hess B (1992) Tamm-Horsfall Glycoprotein. Inhibitor or promoter of calcium oxalate monohydrate crystallisation process? Urol Res 20: 83
10. Knorle R, Schnierle P, Koch A, Buchholz NP, Hering F, Seiler H, Ackermann T, Rutishauer G (1996) Tamm-Horsfall glycoprotein: role in inhibition and promotion of renal calcium oxalate stone formation studied with Fourier-transform infrared spectroscopy. Clin Chem 40: 1739
11. Lewandowski S, Rodgers A, Schloss I (2000) The influence of a high oxalate diet on the risk of calcium oxalate renal stone formation in healthy black and white South Africans. In: Rodgers AL, Hibbert BE, Hess B, Khan SR, Preminger GM (eds) Urolithiasis 2000. University of Cape Town, Cape Town, p 300
12. Medetogon-Benissan J, Tardivel S, Hennequin C, Daudon M, Drueke T, Lacour B (1999) Inhibitory effect of bikunin on calcium oxalate crystallisation in vitro and urinary bikunin in renal stone formers. Urol Res 27: 69
13. Meyers AM, Whalley N, Zakolski WJ, Shar T (1994) Chemical composition of the urine in the normal black and white population. In: Ryall RL, Bais R, Marshall VR, Rofe AM, Smith LH, Walker VR (eds) Urolithiasis 2. Plenum Press, New York, p 422
14. Modlin M (1967) The aetiology of renal stone: a new concept arising from studies on a stone-free population., Ann Roy Coll Surg Engl 40: 155
15. Ryall RL, Grover PK, Stapleton AMF, Barrell DK, Tang Y, Moritz RL, Simpson RJ (1995) The urinary F1 activation peptide of human prothrombin is a potential inhibitor of calcium oxalate crystallization in undiluted human urine in vitro. Clin Sci 89: 533
16. Schwager SLU, Chubb AJ, Scholle RR, Brandt WF, Eckerskorn C, Sturrock ED, Ehlers MRW (1998) Phorbol ester-induced juxtamembrane cleavage of angiotensin-converting enzyme is not inhibited by a stalk containing intrachain disulfides. Biochem 37: 15449
17. Shiraga H, Min W, VanDusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Nielson EG, Hoyer JR (1992) Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Nat Acad Sci USA 89: 426
18. Walz DA, Hewett-Emmett D, Seegers WH (1977) Amino acid sequence of human prothrombin fragments 1 and 2. Proc Natl Acad Sci USA 74: 1969
19. Werness PG, Brown CM, Smith LH, Finlayson B (1985) EQUIL2: a basic computer programme for the calculation of urinary supersaturation. J Urol 134: 1242
20. Whalley N, Moreas M, Shar T, Pretorius S, Meyer A (1998) Lithogenic risk factors in the urine of black and white subjects, Br J Urol 82: 783
21. Worcester EM (1996) Inhibitors of stone formation, Semin Nephrol 16: 474