Urine protein markers distinguish stone-forming from non-stone-forming relatives of calcium stone formers

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A number of proteins in urine have been implicated in the process of calcium kidney stone formation, due to either their ability to inhibit crystallization of calcium oxalate (CaOx) and calcium phosphate stone-forming (SF) salts or their presence in the matrix of these stones. Such proteins include Tamm-Horsfall protein (THP) (20), osteopontin (uropontin) (2), prothrombin fragment 1 (PF1) (38), inter-α-trypsin inhibitor (ITI) (9), and bikunin (BK), a portion of ITI (3), calgranulin (34), CD59 (7), and albumin (10). Although some reports suggest defects, deficiencies, or variations of these molecules among SF vs. normal subjects (13, 14, 26, 27, 43), it remains unclear whether any of these is a cause or a consequence of stones; no research thus far has translated molecular knowledge into clinical practice.

Among the many proteins that have been linked in some way to stone formation, it is possible that some are more strongly associated with nephrolithiasis than others. If this is the case, certain protein measurements may be better at distinguishing SF from none-stone-forming (NS) in a population, using statistical techniques of classification. We wish to identify those proteins that are most strongly associated with stone formation to focus attention on those that may offer the greatest insights into the disease.

Our approach here has been to measure multiple urine proteins in groups of SF and NS subjects, all of whom are first-degree relatives of calcium SF patients. We reasoned that by making our comparisons within SF families, we would reduce the effects of well-known inherited stone causes such as hypercalciuria so that possible links between protein biomarkers and being a SF or not might be more readily observed.

MATERIALS AND METHODS

Study Subjects

During a concurrent study of families with calcium stone disease (National Institute of Diabetes and Digestive and Kidney Diseases Grant R44-DK-54585), 24-h urine samples were obtained from 902 first-degree relatives of calcium SF. From this pool, we chose 100 subjects at random, one-half female, one-half SF by sex to create a balanced block design of 4 cells (male NS and SF, female NS and SF) with 25 subjects/cell. The incidence of stones was self-reported; documentation of stone type was unavailable. We excluded subjects with dipstick proteinuria or glucosuria above trace, to avoid potential effects of renal disease. Also, we included only subjects who were not taking medications that could affect stones or mineral metabolism. These medications included thiazide and other diuretic agents, vitamin D supplements, and alkali supplements.

To select our set of 100, we used a random number table, choosing the first eligible SF in the list that met an arbitrary criterion, an even number. Thereupon, we selected as a NS control the first same-sex subject within 5 yr of age, meeting the second criterion, an odd number on the random table. We continued this process until we had obtained our set of 100, which is a true random sample of the original 902. These 100 subjects arose from 74 families, each of which contained a SF proband. No probands are included here. For 58 of the 74 probands, we had stone analyses or documentation of calcium stones according to their physicians' records. For the remaining 16 probands, we had knowledge that stones were not struvite, cystine, or uric acid, and they were therefore classified as “calcium” SF.

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TABLE 1. Characteristics of subjects grouped by sex and stone status

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No stones</td>
<td>Stones</td>
</tr>
<tr>
<td>Age, yr</td>
<td>50.6 ± 2.3</td>
<td>51.1 ± 2.2</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>85.2 ± 2.6</td>
<td>84.5 ± 1.8</td>
</tr>
<tr>
<td>Protein</td>
<td>402.1 ± 20.7</td>
<td>402.1 ± 25.7</td>
</tr>
<tr>
<td>Calcium</td>
<td>244.5 ± 25.8</td>
<td>263.9 ± 21.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>643.0 ± 59.0</td>
<td>509.2 ± 36.8</td>
</tr>
<tr>
<td>Magnesium</td>
<td>105.8 ± 5.8</td>
<td>109.1 ± 7.7</td>
</tr>
<tr>
<td>Ammonia</td>
<td>37.6 ± 2.9</td>
<td>40.3 ± 2.4</td>
</tr>
<tr>
<td>Sodium</td>
<td>210.9 ± 16.7</td>
<td>187.7 ± 10.5</td>
</tr>
<tr>
<td>Sulfate</td>
<td>24.2 ± 1.7</td>
<td>24.8 ± 1.4</td>
</tr>
<tr>
<td>Oxalate</td>
<td>43.4 ± 3.7</td>
<td>44.5 ± 3.5</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>1,087.6 ± 70.0</td>
<td>1,108.0 ± 65.1</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1,885.9 ± 81.1</td>
<td>1,829.6 ± 59.5</td>
</tr>
<tr>
<td>Uric acid</td>
<td>687.8 ± 51.5</td>
<td>648.0 ± 33.5</td>
</tr>
<tr>
<td>Volume, liters/day</td>
<td>1.87 ± 0.16</td>
<td>1.84 ± 0.15</td>
</tr>
<tr>
<td>Ca/Cr</td>
<td>130.5 ± 11.3</td>
<td>136.6 ± 10.1</td>
</tr>
<tr>
<td>Citr/Cr</td>
<td>343.7 ± 32.2</td>
<td>280.0 ± 22.2</td>
</tr>
<tr>
<td>Urine pH</td>
<td>6.00 ± 0.10</td>
<td>5.80 ± 0.08</td>
</tr>
<tr>
<td>SS CaOx</td>
<td>8.4 ± 0.8</td>
<td>9.4 ± 0.8</td>
</tr>
<tr>
<td>SS CaP</td>
<td>1.4 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>SS UA</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>CG</td>
<td>40.2 ± 1.2</td>
<td>42.4 ± 1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. All subjects are 1st degree relatives of calcium stone formers. Units for protein, calcium, citrate, magnesium, oxalate, phosphorus, creatinine, and uric acid are mg excreted daily; ammonia, sodium, and sulfate are mmol/day; and Ca/Cr and Citr/Cr are calcium (in mg) excreted daily per g creatinine and citrate (in mg) excreted daily per g creatinine, respectively. SS CaOx, SS CaP, SS UA are calculated supersaturations for calcium oxalate, calcium phosphate and uric acid, respectively; CG is %control crystal growth rate, a measure of CAOx crystal growth inhibition. Stone formers and non-stone formers do not differ significantly with respect to any of the measurements.

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with a donkey anti-sheep IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:10,000. Chemiluminescence of the ECL substrate was detected by digital image acquisition using a cooled CCD camera system (BioChem System, UVK, Upland, CA). Exposure time was 1 min. Antibodies were stripped from the membrane using Restore Western blot stripping buffer (Pierce Biotechnology) according to the manufacturer’s instructions. A second Western detection was then performed as above by incubating the membrane with rabbit polyclonal antibody to human inter-α-trypsin inhibitor (A0301, Dako, Carpentaria, CA) diluted 1:2,000 followed by goat anti-rabbit IgG secondary antibody at 1:10,000 (all from Santa Cruz Biotechnology) with an exposure time of 1 (calB) or 10 min (CD59). All secondary antibodies (as purchased) were preadsorbed with human serum proteins to minimize cross reactivity to other proteins in urine.

Primary antibodies used for the detection of THP, albumin, α-1-microglobulin, and calgranulin A (calA) were sheep anti-human THP (Cortex Biochem, San Leandro, CA), sheep anti-human albumin (Biodesign International, Saco, ME), sheep anti-human α-1-microglobulin (Biodesign International), and goat anti-human calA (Santa Cruz Biotechnology). Appropriate secondary antibodies and the same dilutions were used as above.

**Data Analysis**

**Image analysis.** Digital images were stored in established quantitation software (LabWorks, UVK). By lane, each image was processed to measure location and intensity of all bands. Location was in molecular mass units taken from conventional standard lanes. The optical density (OD) of each band and the total OD of the lane were determined by the software, then intensities of each protein were calculated in three ways. First, the OD of each band was divided by the total OD of the lane to yield a unitless ratio that expresses the OD of each band as a percentage (p) of the total for that individual sample. The OD fractions of the bands added up to 100% for each person, but mean values for the individual groups of men and women, with and without stones do not add up to 100%, as expected, because means of ratios are not equal to ratios of means. Second, the OD of each band was divided by the total OD of a standard urine sample lane that was run on every gel. This semiquantitative corrected OD can be used to compare abundances of each band between samples and gels in absolute (a) terms. Finally, the total OD in the lane was divided by the total OD of the standard lane to express total (t) immunoreactivity for the protein as a fraction of that of the standard urine.

**Statistical analysis.** Molecular mass and intensity data were ported to standard statistical software (Systat 11, Systat Software, Richmond, CA) and merged with panels of age, sex, SF status, crystal growth inhibition, and stone risk urine chemistry data. We chose age and sex as demographic demarcators because both are well known to have effects on mineral metabolism and prevalence of stone disease. The association of individual variables with stone formation was estimated using a combination of simple univariate t-tests and multivariate discriminant analysis. Our strategy was to maximize discovery of possible protein relationships to stone forming. To do this, we essentially screened all protein measurements for differences by sex or stones using t-tests. As a more stringent screen, we performed ANOVA by protein in which protein abundance was the dependent variable with sex and SF as factors. Where ANOVA showed sex or stone-sex interactions of significance (P < 0.05) we performed post hoc hypothesis testing by sex as appropriate. To preserve both analyses, we show significance values by t-test as superscripts and those confirmed by ANOVA as bold superscripts (see Table 2). Finally, we included all proteins we measured as candidates in multivariate discriminant analysis.

**RESULTS**

**Electrophoretic Profiles of Urine Proteins**

**PF1.** By visual inspection of Western blots, a PF1 band with a molecular mass of 31 kDa predominated among most subjects (Fig. 1A). Many samples also contained a lower molecular mass (LMM) form of ~23 kDa. Less abundant higher molecular mass forms (HMM: >40 kDa) of PF1 were also visible in some samples. A few samples also contained smaller immunoreactive bands (~20 kDa) that are probably products of proteolytic degradation of PF1.

**III.** BK migrated as a prominent band at ~37 kDa, in agreement with the 35- to 40-kDa size of native urinary BK
(Fig. 1B) (17). Most samples contained a second band of ~21 kDa. Weakly staining HMM bands (>50 kDa) were visible in most samples, and a small portion contained LMM bands (<20 kDa).

CD59. CD59 migrated principally as a band of ~20 kDa, consistent with the molecular mass of 18–20 kDa observed by others using SDS-PAGE (Fig. 1C) (29). In addition, samples from several subjects contained LMM bands, which may be either products of deglycosylation (28) or proteolytic cleavage.

CalB. CalB Western blots (Fig. 1D) showed a major immunoreactive band at 14 kDa that is the monomeric form of this protein (31). In addition, forms of ~30 and ~40 kDa were also apparent. Samples from several subjects contained LMM bands (<12 kDa) that are likely to be proteolytic cleavage products of calB (not shown).

Other proteins. Similar Western blots were prepared to analyze other proteins of interest, including THP, albumin, α-1-microglobulin, and calA. No significant differences were detected in the urinary abundances of these proteins between SF and NS, and none were informative in any of our analyses; thus the blots are not shown.

Univariate Analysis of Isoform Abundances

Differences between SF and NS. For clarity, we repeat here that we use “p” to denote the proportion of immunoreactivity ascribed to a single isoform, “a” to denote the abundance of the isoform compared with a standard, and “t” to denote the total ascribed to a single isoform, “a” to denote the abundance of the isoform, “p” to denote the proportion of immunoreactivity relative to total OD of a standard urine control, or total (t) lane OD normalized to the control lane. Differences by univariate t-test: * vs. no stones, same sex, P = 0.025; ** vs. no stones, same sex, P = 0.012; $ vs. no stones, same sex, P = 0.005; † vs. no stones, same sex, P = 0.002; ‡ vs. no stone men, P < 0.004; ¶ vs. no stone men, P < 0.001; ¶‡ vs. no stone men, P = 0.026; ‡‡ vs. no stones, same sex, P < 0.05; ¶‡ vs. stone-forming men, P < 0.004; ¶‡ vs. stone-forming men, P < 0.01; ¶‡ vs. no stone men, P = 0.021. Differences confirmed by ANOVA are indicated by bold superscripts.

For protein analysis, we included all measurements of every protein (including PF1, ITI, CD59, calA, calB, THP, albumin, and α-1-microglobulin) in the initial model. From this set, HMM PF1, HMM ITI, BK, and HMM and low-molecular-mass (LMM) forms of calB (Fig. 2) classified 84% of male subjects correctly (P = 0.0008). The false-positive and false-negative results were both low, at 16%. In women, CD59, BK, and HMM ITI correctly identified 76% of subjects (P = 0.003), with false-positive and false-negative rates of 24%. Although the sets of proteins contributing to classification were not the same for both sexes, BK and HMM ITI forms were common discriminators. Interestingly, entry of BK into either model does not appear to improve the results (Fig. 2), but analyses performed without this variable failed to achieve more than marginally significant results, indicating a complex relationship with other discriminating proteins.

Multivariate Analysis of Proteins in Relation to SF

As expected from Table 1, conventional RF were not useful in distinguishing SF from NS by multivariate discriminant analysis. When all subjects were considered together, the results were insignificant, with 55% of subjects correctly classified by stone status and only urine pH entering the model (P = 0.14). In men alone, citrate excretion and supersaturation of CaOx (SS CaOx) entered the model, but the overall success rate of classification was rather low (70%), and the P value was marginally significant (P = 0.05). In women, analysis by forward stepping failed to produce any classification by RF, as no variables entered into the model.
DISCUSSION

Our results point to HMM ITI as a potential urine protein correlate of stone formation within families of probands who were themselves calcium SF. The ITI family of proteins consists of three heavy chains (H1, H2, H3) of 75- to 85-kDa molecular mass, and one light chain, BK, with a molecular mass of 35–40 kDa (17, 39). BK can be linked covalently through its chondroitin-4-sulfate side chain to one or two heavy chains, forming the pre-α-inhibitor (PαI; H3 + BK, 125 kDa) or ITI (H1 + H2 + BK, 180–220 kDa) (8, 11, 36), also known as ITI-trimer (26). The >50-kDa bands we detected here most likely correspond to heavy chain components, PαI or ITI. The 21-kDa BK form we found corresponds to the ~25-kDa BK form identified by Suzuki et al. (43) as deglycosylated BK or a 20-kDa fragment of BK, also known variously as urinary trypsin inhibitor 2 (UTI-2) (15, 22) or uristatin (37). LMM bands (<20 kDa) are products of proteolytic degradation.

In a less striking way, BK measurements contributed to discrimination in all subjects as well. Men and women differed in the types of HMM ITI and BK measurements entering the classification, whether a proportion (p) of immunoreactivity in that protein form within a sample or an absolute (a) amount relative to a standard urine control. The reason for this sex difference is not clear, but in any case HMM ITI and BK measurements were important discriminators.

There are conflicting reports in the literature regarding the relationship between ITI and stone disease. Medetognon-Benissan et al. (27) reported that BK levels were lower in SF than in healthy controls. By contrast, Suzuki et al. (43) found higher urinary concentrations of BK in male SF compared with healthy male controls and that a greater proportion of SF than normal patients had an aberrant BK 25-kDa species, which is identified to be deglycosylated BK, a form with reduced effectiveness in inhibiting CaOx crystal growth. Marengo et al. (14, 26) found increased HMM forms (PαI and ITI), but not BK, in SF (14, 26), and these differences were constant over several months (26).

Prothrombin fragments were strong predictors of stone forming in men. The abundant 31-kDa protein we observed is consistent with urinary prothrombin fragment 1, formerly referred to as crystal matrix protein (40). The 23-kDa form corresponds to either a thrombin-derived cleavage product of PF1 (33) or deglycosylated PF1 (35). HMM forms (>40 kDa) are likely to be other PF1-containing activation peptides of prothrombin, primarily prothrombin fragment 1+2, with a molecular mass of 43 kDa (40). Immunohistochemical analysis of human kidneys has shown that PF1 is synthesized in the proximity of renal stones and is more abundant in the kidneys of SF than in healthy individuals (41). However, to date no differences have been found in the relative amounts of any prothrombin forms in the urine of CaOx SF vs. normal controls (14).

PF1 and BK are potent inhibitors of CaOx crystal growth (3, 4, 38), and can be produced in the kidney (30, 42). While the entire ITI molecule has been shown to inhibit CaOx crystallization (9), the heavy chain components do not have inhibitory activity, and BK is thought to be responsible for the ability of ITI to inhibit CaOx crystallization (21). Interestingly, we observed here that urine from women contains a lower proportion of intact 31-kDa PF1 and less BK and total ITI than that from men. Such differences may well be a reason that we found reduced whole urine crystal growth inhibition among women vs. men (6).

In women, but not men, the amount of CD59 was successful in discriminating SF from NS. Little is known about the relationship of CD59 to stone disease. CD59 is an 18- to 20-kDa glycoprotein inhibitor of the membrane attack complex of complement that is thought to play a role in protecting the kidney from complement-mediated injury (47). CD59 deficiency and increased urinary excretion of CD59 have been linked to renal tissue damage (18, 24, 45). CD59 is an acidic protein with a modest ability to inhibit CaOx crystallization (Coe F, unpublished observations), but its possible association with nephrolithiasis has not been otherwise investigated.

Calgranulin is a strong inhibitor of CaOx crystal growth and consists of two monomeric subunits, A and B (also known as MRP8 and MRP14, or S100A8 and S100A9), which are small (11 and 14 kDa, respectively) acidic proteins that can combine to form homo- and heterodimers (24–28 kDa), as well as tetramers and other higher-order multimers (35–48 kDa) (16, 31, 34, 44). In fact, it is the calA/calB heterodimer for which crystal growth inhibition has been documented (34). Although the heterodimer is thought to be the functional form of the protein, there is increasing evidence that the monomers and
other conformational forms may exert individual functions (31). We observed protein bands with sizes corresponding to monomeric, dimeric, and multimeric forms on both our calA and calB blots. However, we found that only calB-immunoreactive forms, but not calA, contributed to the discrimination of SF from NS in men. The relevant calB bands were in the HMM (>40 kDa) region of the gel, which may represent multimeric forms of the protein. However, it is not clear whether such forms would be expected to be visible under the denaturing and reducing conditions used here, as the proteins are noncovalently linked, so the identity of these bands is uncertain (44). LMM calB (<12 kDa), which also entered the classification model, most likely represents proteolytic cleavage products of various calB forms.

In addition to inhibiting CaOx crystallization, ITI, PF1, CD59, and calgranulin are known to have roles in mediating inflammation or cell injury and repair, and their role in the pathophysiology of nephrolithiasis may not be limited to or even involved with crystal inhibition (46). Expression of these proteins may be provoked by renal tissue damage from stones or crystals. Alternatively, these proteins may promote stone formation by acting as nucleators or as a matrix for crystallization (49). In any case, stone disease may induce changes in expression or posttranslational modification of PF1, ITI, CD59, and calgranulin in the kidney, which could consequently affect the form or abundance of these proteins in urine. We may well be observing kidney cell responses to the presence of stones or crystals, or perhaps injuries from stone-passage events.

The source of these proteins in urine is not yet certain, as they could be filtered, in part, from blood. However, HMM ITI forms, which were found to be important predictors of stone formation, would not be electropheretically filtered due to their size. This suggests that HMM ITI in urine is of renal rather than hepatic origin (17, 26). What does seem reasonable, given the present work, is that these questions concerning the source and purpose of urinary ITI, PF1, CD59, and calgranulin are significant issues for subsequent research.

Technically, this study is limited by the electrophoretic conditions used to separate the proteins and the semiquantitative nature of the measurements. We can be reasonably sure that the PF1, ITI, CD59, and calB bands we have observed represent the molecular species that we have discussed in this report. However, the conditions we used here were initially chosen for the analysis of LMM proteins such as calgranulin and were not ideal for resolution and quantitation of HMM forms (>50 kDa).

Proteolysis could produce a shift from HMM to LMM species and be more prominent in SF than NS. Bautista et al. (5) found aberrant LMM OPN variants more frequently in SF than normal controls due to increased serine proteases in their urine. We have not observed that urine PF1, ITI, CD59, or calgranulin is susceptible to rapid degradation in the absence of protease inhibitors (data not shown). In any case, protease degradation is not a likely explanation for what we observe with ITI, because we do not detect the appearance of new aberrant LMM forms but rather an increase in the abundance of 37-kDa BK and HMM ITI forms in the urine of SF.

This study was designed so as to reduce differences of standard urine RF between SF and NS. Because all subjects were first-degree relatives of calcium SF, there was no difference in urine calcium levels between SF and NS here, whereas we (32) and others (25) have found higher urine calcium levels among SF vs. normal people. We chose these conditions to reduce the effects of routine factors such as urine calcium to see the effects of urine proteins against a more neutral background.

In conclusion, this study supports the hypothesis that variations in the abundance and electrophoretic mobility of specific urine proteins are associated with stone formation. In these relatives of calcium SF, PF1, ITI, CD59, and calB isoforms are candidates for identifiers of SF. The meaning and origin of the sex differences in these proteins, and the differences between SF and NS are not clear. However, even so, PF1, ITI, CD59, and calB do seem either to affect stone disease, respond to it, or, at least, be somehow related to stones through some intervening mechanisms. These molecules have great potential, as markers and predictors of SF propensity, or as clues to the development of new drugs for prevention of stones or renal crystallization. Given our own recent proofs that CaOx SF deposit interstitial apatite crystals in their inner medullary tissues (12), understanding and exploiting the properties of crystal inhibitors may well be of even greater clinical importance. If future studies show that cosegregation is robust and reproducible, it will be difficult to accept the null hypothesis that such proteins have no role in stone disease, as a cause of stones, or as a consequence.

ACKNOWLEDGMENTS

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DISCLOSURES

Brian Coe and Fredric Coe have financial interests in Litholink Corporation and are on the board of directors.

REFERENCES

URINE PROTEIN MARKERS IN NEPHROLITHIASIS


