Proteomic analysis of renal calculi indicates an important role for inflammatory processes in calcium stone formation

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RENAI STONES affect 5% of the female and 12% of the male US population and result in an average of 19 hours lost work per person, costing an average of $3,500 per incident (5, 25, 32, 35). Several mechanisms for a range of renal stone types have emerged over the past three decades (5). On the basis of frequency of occurrence, renal stones are most prevalently composed of calcium oxalate (CaOx). Simplistically, an initiating event in the formation of CaOx stones is the precipitation of ionized calcium and oxalate out of supersaturated urine to form calcium oxalate monohydrate (COM) crystals. This precipitation process is, however, complex and governed by many factors including specific urinary proteins. Specific protein incorporation into the COM crystal lattice (and perhaps thereby calcium stones) can occur in a cell-free system, thus implying that the molecular structure of the biomolecules is sufficient to determine whether or not the biomolecules bind and are incorporated into the mineral matrix (24, 31). Proteins such as Tamm-Horsfall protein (THP) and osteopontin (OPN) have been reported to act as modulators of the formation as well as the aggregation of COM crystals (4, 20, 28, 33), a result perhaps dependent on amino acid composition or posttranslational modification (4, 6, 13, 33). A prevailing theory of CaOx stone formation, the fixed particle theory, dictates that COM crystals bind to the membranes of damaged or proliferating renal tubular epithelium (tubular nephrocalcinosis) or within the renal papilla suburothelium (interstitial nephrocalcinosis) (21, 37). Unique preexisting features, such as injured or proliferating cells or calcium phosphate (CaP) crystals localized to the suburothelium of renal papilla, facilitate the COM crystal binding. COM crystal binding to renal tubular epithelial cell membranes is toxic to the cell, inciting an inflammatory response (18). COM crystal aggregation and stone formation develops in preexisting renal papillary lesions containing microroles of CaP, referred to as Randall’s plaques (8, 10). In either tubular or interstitial nephrocalcinosis, CaOx stone growth can incite cellular inflammatory reactions and recruitment of inflammatory cells (17, 18), which can feasibly contribute to and modify the urinary, crystal, or stone proteome.

In addition to the contribution of inorganic and organic ions toward CaOx stone formation, the presence of specific urinary biomolecules (proteins and glycosaminoglycans) plays an important role in crystal aggregation and thus stone formation (30). Some proteins such as THP, soluble OPN, prothrombin fragment 1 (PF1), bikunin, inter-α-trypsin inhibitor, α₁-microglobulin, calgranulins, fibronectin, and matrix gla protein have been shown to inhibit crystal aggregation and hence stone formation (19), while other urinary proteins are associated with stone formation in humans (2, 9). Several investigators have examined the protein content of CaOx stones, identifying proteins either coating the crystals or embedded in the stones’ crystalline structure such as OPN, THP, and urinary PF1 (22). The significance of these differences in protein association with stones and their roles in inhibition, promotion, or reaction to stone formation, while heavily investigated, remain to be thoroughly defined. In addition to the contribution of constitutive urinary proteins, the elicited inflammatory responses may further contribute to the proteomic milieu. These proteins, whether from cellular compartments of the kidney or from filtered serum proteins, will all likely contribute to the composition of the stone proteome. Our results here identify previously unreported stone matrix proteins (SMP), some of which may be involved in critical elements of stone formation. Further analysis of our identified SMP demonstrates groups of...
related proteins, some of which are associated with inflammatory responses and further support a hypothesis that aspects of stone formation or propagation may involve a cellular inflammatory contribution.

METHODS

Protein isolation. Renal stones were collected from five individuals under a University of Louisville Institutional Review Board-approved waste sample study protocol. Stones were demineralized and SMP were isolated with modifications of the procedures of Doyle et al. (7).

One-dimensional polyacrylamide electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis. For indirect mass spectrometry (MS) analysis, a SMP sample from one individual (10 μg) was separated with one-dimensional electrophoresis on a 10% NuPAGE gel (Invitrogen, Carlsbad, CA) and stained with colloidal Coomassie blue, and visualized protein bands were identified as described previously (1) with a modification of the procedures of Jensen et al. (16).

Liquid chromatography and electrospray ionization-tandem MS analysis. For direct MS analysis a protein (10 μg) isolated from the stone samples of three different individuals was trypsinized with a modification of the procedures of Jensen et al. (16). The tryptic peptides were concentrated and desalted with a reverse-phase (RP) cartridge (Michrom BioResource, Auburn, CA) and separated by capillary RP chromatography column (10 cm of Synergi 4-μm RP80A Phenomenex). Spectra were acquired with a LTQ linear ion trap mass spectrometer (Thermo Electron). During liquid chromatography (LC)-tandem MS (MS/MS) analysis, the mass spectrometer performed Xcalibur-assisted data-dependent acquisition with a full MS scan between 300 and 2,000 m/z followed by three MS/MS scans (35% exclusion duration window).

MS data analysis. The acquired matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) MS/MS data were searched against a Swiss Prot human protein database (Human-RefSeqXPs) from an NCBI nonredundant database with the Mascot v2.1, assuming static modifications for sodiation of asp and glu, hydroxylation for pro, lys, and asn, and carbamidomethylation of cys. The acquired LC-electrospray ionization (ESI)-MS/MS linear ion trap data were searched against a translated human genome database (Human-RefSeqXPs) from an NCBI nonredundant database with the Sequest algorithm, assuming carbamidomethylation (+57) of cys; in all cases variable oxidation of met (+15.99) was considered.

Database analysis of ion trap data was performed with Sequest Sorcerer (Sage-N Research, San Jose, CA). High-probability protein identifications were assigned from the Sequest results with the ProteinProphet (http://tools.protemecenter.org/software.php), which uses expectation maximization and discriminant analysis to separate real assignments from incorrect assignments. ProteinProphet also eliminates redundancy within spectral groupings by assigning proteins with 100% identity (based on shared MS/MS spectra) (27). The MS/MS files and protein assignment data were fitted to a receiver operator curve, and proteins with a false positive error of <10% were accepted as correct assignments. Spectra data files generated by Sequest Sorcerer (SageN) were submitted to BIGCAT software and BIGCAT filter uses Sequest Xcorr cutoffs of 1.5, 2, and 2.5 for +1, +2, and +3 ions, respectively. The BIGCAT software also establishes a protein abundance factor (PAF) for comparison of protein enrichment within and between experiments (29) (Supplemental Methods).

RESULTS

SMP analysis by MS methods. Comparable amounts of protein (10 μg) were used for SMP analysis by either one-dimensional (1D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with MALDI-TOF MS identification or 1D RP LC-MS analysis. Few proteins were identified from the SDS-PAGE separation. SMP 1D-SDS PAGE (Fig. 1) analysis resulted in 12 protein bands. Four proteins (OPN, albumin, vitamin K-dependent protein Z, and PF1) were identified as components of five bands. Of note, multiple protein bands positively identified as OPN but migrating at molecular mass

Excel tables containing accession (gi) numbers and gene names for all high-probability Protein/PeptideProphet-identified proteins were constructed. These data tables were submitted for gene ontology analysis (AmiGO; http://amigo.geneontology.org) to define respective protein cellular origin and for bioinformatic pathways analysis [Ingenuity Pathway Analysis (IPA), Ingenuity Systems, Mountain View, CA] to define functional patterns within SMP proteome composition.

Immunoblot analysis of SMP. Ten proteins were selected for immunoblot (IB) validation using methods as previously described (1) to confirm single peptide protein identification and to define protein fragment identity. IBs were performed for 1) OPN, 2) nucleolin (NCL), 3) myeloperoxidase (MPO), 4) heat shock protein (HSP)(90α/β, 5) superoxide dismutase 3 (SOD3), 6) histone H4 (H4), 7) nucleophosmin (NPM), 8) HSP27, 9) myosin heavy chain-9 (MYH9), and 10) lactotransferrin (LTF) with appropriate secondary antibody (horseradish peroxidase) conjugates.

Fig. 1. Composition of the stone matrix proteome is dominated by a limited number of high-abundance proteins and protein fragments. Analysis of stone matrix proteins (SMP) with 1-dimensional electrophoresis (IDE) and matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) identified a relatively low number of high-abundance proteins and putative protein fragments.

1 The online version of this article contains supplemental material.
Stone matrix protein (SMP) proteins were identified by direct analysis of protein tryptic fragments with liquid chromatography-mass spectrometry and Protein/Peptide Prophet for data analysis. Common SMP (bold) were defined as being identified in all 3 analyses. Prevalent SMP were defined as being identified in 2 of 3 SMP analyses. Protein abundance was estimated with the protein abundance factor (PAF) scoring generated by BIGCAT software. PAF scoring is based on the number of peptides observed for a specific protein and normalized for the cognate protein’s molecular weight. *Proteins previously acknowledged as proteins binding to calcium oxalate or apatite surfaces (3, 14, 26, 30, 34, 36).

Table 1.—Continued

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Stone matrix protein (SMP) proteins were identified with gel electrophoresis and MALDI-TOF MS. Twenty-eight proteins (Table 1, bold) were detected in all three LC-MS stone analyses. These common proteins included apolipoproteins, myeloid lineage-specific proteins, and calcium binding proteins. Apolipoproteins identified in all three LC-MS analyses included apolipoprotein A-I, apolipoprotein A-IV, apolipoprotein D, apolipoprotein E, and apolipoprotein J (clusterin). Apolipoprotein B was identified in two of the three LC-MS runs. Myeloid lineage-specific proteins identified here included S100A9, S100A8, SET translocation, LTF, and MPO and were identified in all three LC-MS stone analyses. Calcium binding proteins identified included S100A8, S100A9, THP, coagulation factor II, vitamin K-dependent protein Z, serum amyloid P, MYH9, MPO, mannan-binding lectin serine protease 2, coagulation factor X, fibrinogen, arylsulfatase family member, OPN, and SPP2. Fifty-eight prevalent proteins were detected in at least two of the three LC-MS stone analyses (Table 1).

Label-free quantification of SMP abundance. With the use of AmiGO for gene ontology analysis, the origins of the 28 common proteins were assigned as 61% extracellular, 36% intracellular, and 3% intra-/extracellular. The origins of the 58 prevalent proteins were assigned as 48% extracellular, 45% intracellular, and 7% integral membrane. On the basis of the normalized abundance of the peptides identified by MS/MS (PAF scores >2.0), four proteins (S100A8, S100A9, apolipoprotein A-I, THP) were categorized as prevalent, high-abundance SMP. Ten proteins [albumin, OPN (secreted phosphoprotein I), thrombin (coagulation factor II), vitamin K-dependent protein Z, apolipoprotein E, SET translocation, histidine-rich glycoprotein, arginine-serine-rich 3 splicing factor, RPM1, vitronectin] were categorized as prevalent, medium-abundance SMP (PAF scores 0.5–2.0). The remaining 14 proteins [serum amyloid P, apolipoprotein A-IV, apolipoprotein D, clusterin (apolipoprotein J), keratin 7, keratin-14, LTF, MPO, SOD3, HSP90α/β, MYH9 and kazarin] were categorized as prevalent, low-abundance SMP (PAF scores <0.5).

Ingenuity Pathway Analysis of SMP functions and origins. IFA of the 28 common SMP identified three protein interaction networks with the highest probability network being associated with tumorigenesis, lipid production and transport, and small molecule biochemistry. This high-probability network involved 36 gene products and has a score for random observa-
tion of 1 out of $10^{45}$. Thus the chance of these proteins being randomly identified together in one analysis was highly unlikely (1 out of $10^{45}$). Fully half of the proteins populating this protein network (albumin, apolipoproteins D, E, J, fibrinogen, HRG, HSP90α/β, keratins 7 and 14, LTF, NPM, S100A8 and A9, OPN, and vitronectin) were observed in these MS analyses. Pathways analysis of the 28 common SMP identified three principal diseases including 1) tumorigenesis, 2) immunological disease, and 3) inflammatory disease. The top canonical pathway defined with the biochemical roles of these 28 common proteins was the acute-phase response signaling pathway. IPA analysis of all 58 prevalent proteins yielded results comparable to the common matrix proteins.

Validation of SMP expression. MS-determined protein expression was confirmed by IB for five proteins including known COM crystal binding proteins (OPN and NCL), myeloid lineage-specific proteins (MPO and LTF), and also HSP90α/β (a protein whose MS identification is based on a single peptide), and details are presented in Fig. 2, A–E. The IB experiments for the remaining five proteins (SOD3, H4, NPM, HSP27, and MYH9) were not successful, and no immunopositive bands were demonstrated on developed film. Therefore these data do not support the presence or the absence of these specific proteins in the SMP.

DISCUSSION

The pathogenic mechanisms of renal calculi formation are complex. None of the ~80 known SMP has been shown to play the decisive role in stone formation, nor do they adequately explain the many suggested mechanisms for stone formation (2, 3, 30). The >70 new SMP we have identified here add to this proteome complexity. On the basis of label-free MS analysis many of these proteins were of low abundance and in some instances identified by single peptides observed across multiple samples. Our acceptance of these data is increased and supported by the IB validation of HSP90α/β (Fig. 2F), a protein identified with LC-MS by a single peptide. In total, these protein identifications, generated from a small number of stones, represent a significant increase in the known stone matrix proteome. These data may prove useful in understanding the roles of genetic susceptibility (2) and specific proteins in stone formation (12).

The array of identified SMP parallels the complexity of stone formation. The large number of calcium binding proteins presented here confirms the role of divalent cation binding proteins in calculus formation. A limited set of membrane-associated proteins including CD180, prosaposin isoform a, kazrin isoform A, clusterin, syndecan 4, and NCL support the importance of cell membranes in general as participating in calculus formation as previously described by others (10, 18, 21, 23, 37). Interestingly, a known NCL binding partner, MYH9, was identified here by MS methods as a component of the SMP. NCL and MYH9 were previously shown to cotranslocate to the surface of endothelial cells after the cell’s adherence to extracellular matrix components and vascular endothelial growth factor stimulation (15). While it is impossible to construe from these data whether these proteins are derived from distinct cellular processes or are a component of the urinary milieu, these data support the importance of cell membrane and cell turnover in calculus formation. IPA, used to help build relevant candidate protein interaction pathways, inferred that a large number of SMP were associated with inflammation, immune response, and acute-phase response pathways. The relative abundance of numerous myeloid lineage-specific proteins identified here leads us to hypothesize that SMP can originate from the surrounding inflammatory cells recruited by the calculus formation. Finally, the observation of MPO fragments in this work and others (26) and of OPN fragments is consistent with recent work from Grover et al. (12) suggesting that proteolytic activity actively contributes to the stone’s proteome. This proteolytic activity could possibly be associated with the granular components of inflammatory cells.

In conclusion, this proteomic analysis of a limited set of renal stones has significantly expanded the list of known SMP. The diverse origin of these proteins (i.e., extracellular, intracellular, membrane proteins) attests to a complex and multifactorial pathogenesis for stone formation. Our data do not provide discrete mechanistic information regarding how stones form. Indeed, a perhaps significant portion of the identified SMP may be derived from the cellular damage resulting from stone growth and may not involve per se directly with stone growth. However, unbiased pathways analysis of these proteins does suggest that discrete biological processes relating to
inflammation or acute-phase response occur coincident with renal calculus formation. Whether these processes are pathogenic or secondary to stone formation remains to be determined.

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REFERENCES