A Multi-Site Study Confirms Abnormal Glycosylation in the Tamm-Horsfall Protein of Patients With Interstitial Cystitis

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Abbreviations and Acronyms

2-AB = 2-aminobenzamide

DMB = 1,2-diamino-4,5-methylene dioxybenzene

HPAEC = high pH anion exchange chromatography

HPAEC-PAD = HPAEC with pulsed amperometric detection

HPLC = high performance liquid chromatography

IC = interstitial cystitis

MALDI-TOF = matrix assisted laser desorption/ionization-time of flight

MS = mass spectrometry

m/z = mass-to-charge ratio

THP = Tamm-Horsfall protein

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Purpose: We confirm the single site observation of decreased sialylation and abnormal glycosylation of Tamm-Horsfall protein in patients with interstitial cystitis compared to control subjects.

Materials and Methods: Urine samples from 41 controls and 48 patients with interstitial cystitis from a total of 5 North American sites were obtained in blinded fashion as to participant status. Tamm-Horsfall protein was isolated from urine samples by salt precipitation. Protein content was determined by size exclusion chromatography and normalized to creatinine. Sialic acid was quantified by 1,2-diamino-4,5-methylene dioxybenzene (Sigma®) high performance liquid chromatography with fluorescence detection. Neutral and amino sugars were determined by high pH anion exchange chromatography with pulsed amperometric detection. N-glycans were labeled with 2-aminobenzamide and profiled using high pH anion exchange chromatography with fluorescence detection. Samples were also analyzed by matrix assisted laser desorption/ionization-time of flight mass spectrometry. Permethylated N-glycans were analyzed in the mass-to-charge ratio range of 3,000 to 6,000.

Results: There was no difference in the protein-to-creatinine ratio of Tamm-Horsfall protein from patients with interstitial cystitis vs controls (49.12 vs 46.4 mg/gm, $p = 0.26$). Sialic acid content (67 vs 77 nmol/mg Tamm-Horsfall protein, $p = 0.025$) and total monosaccharide content (590.9 vs 680.6 nmol/mg Tamm-Horsfall protein, $p = 0.003$) were significantly decreased in patients with interstitial cystitis vs controls. Results were supported by 2-aminobenzamide N-glycan profiling and mass spectrometry, which showed a 45% decrease in a major tetra-sialylated peak (mass-to-charge ratio 4,590) in Tamm-Horsfall protein from patients with interstitial cystitis compared to controls.

Conclusions: These multisite data validate that abnormal glycosylation of Tamm-Horsfall protein occurs in patients with interstitial cystitis and may have a role in interstitial cystitis causation.

Key Words: urinary bladder; cystitis, interstitial; Tamm-Horsfall protein; glycosylation; N-acetylneuraminic acid

TAMM-HORSFALL protein, which is synthesized in the kidney and is also known as uromodulin, is the most abundant protein in human urine.[1] THP is approximately 30% sugar by weight and it has 8 potential N-glyco-

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sylation sites, which are glycosylated with various di-antennary, tri-antennary and tetra-antennary N-glycans.[2,3]

Its presence in all vertebrate species[4] suggests an important function in the urinary tract but no conclusive evidence to that effect has been reported. It was suggested that THP prevents urinary infection[5] and it has a role in calcium oxalate stone formation.[6]

Another hypothesis is that THP is a protective macromolecule that binds potentially injurious urinary solutes before they can bind to and inflict damage on the urothelial surface of the bladder.[7–9] Earlier studies showed that normal urine contains low molecular weight cationic compounds that injure bladder epithelium in vivo and are also toxic to cultured urothelial cells.[8,9] The healthy bladder must have mechanisms in place to ensure that its protective mucous layer remains intact and potentially harmful substances are confined to the urine until they are voided. Anionic THP sequesters and neutralizes cationic urinary toxic factors.[8,9] Also, THP from healthy individuals is significantly more protective than that from patients with IC against the known cytotoxic effects of the cation protamine sulfate.[9–11]

The protective biological activity of THP depends on the sialic acid present in the protein.[12] In a prior study the sialic acid content of THP from patients with IC at our site was significantly decreased. To confirm those findings we performed the current study, in which samples from patients with IC and control subjects from 5 North American sites were collected and sent in blinded fashion to a laboratory for analysis.

MATERIALS AND METHODS

Urine samples were obtained from patients with IC and controls at a total of 5 North American sites. No patients were from University of California-San Diego, which was the THP source in a previous study. Approximately 1 l fresh urine specimen was obtained per individual. Specimens were shipped cooled but not frozen with 0.01% sodium azide as a preservative. Urine specimens were delivered within 2 days to the laboratory, where all personnel were blinded as to whether samples were from patients with IC or controls.

Selection criteria for controls were a score of 1 or less on the pelvic pain, urgency and frequency questionnaire,[13] and no history of bladder infection, IC, overactive bladder, bladder symptoms, dyspareunia, gynecologic pelvic pain, vaginitis or vulvodynia. Patients met all of National Institute of Diabetes and Digestive and Kidney Diseases clinical criteria for IC (that is no cystoscopy),[14] scored a minimum of 15 points on the pelvic pain, urgency and frequency questionnaire, and had at least a 1-year history of continuous bladder symptoms, including frequency, defined as 10 or more voids in 24 hours, urgency and pelvic pain not due to a gynecologic cause. Urinalysis results in patients with IC were normal and negative for infection.

THP Isolation From Urine

THP was isolated from urine via the salt precipitation method of Tamm et al.[15] Urine was centrifuged for 2 minutes at 3,200 × gravity to remove crystalloid and other debris. It was then incubated in the presence of 0.58 M NaCl overnight at 4C, followed by centrifugation at 3,200 × gravity for 60 minutes. The THP pellet was rinsed twice with 0.58 M NaCl and resuspended in Milli-Q® water. This protein was desalted by ultrafiltration using an Amicon® Ultra 50 kDa cutoff filter. Desalted protein was lyophilized and stored dry at −20C. The purity of the resulting THP was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The quantity of protein used in all assays was determined by weighing lyophilized THP on an XS105 balance (Mettler Toledo, Columbus, Ohio), which is accurate to 0.01 mg.

Protein Measurement

Standard preparation. The THP standard obtained by the salt precipitation method was dried overnight in a vacuum desiccator. Stock solution (2 mg/ml) was prepared in 0.02 M sodium phosphate buffer containing 4 M urea (pH 6.8). For a standard curve a series of dilutions were done to obtain 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156 and 0.008 mg/ml stock solutions. A 100 μl sample was injected onto HPLC.

Sample preparation. THP samples were obtained by ultrafiltration of 10 ml crude urine using an Amicon 50K filter. Samples were centrifuged at 3,200 × gravity for 15 minutes to decrease volume to less than 0.5 ml. To wash the concentrated protein Milli-Q water was added to the THP pellet to bring the volume up to 15 ml and the sample was centrifuged as described. The wash step was repeated another 2 times. The pellet was then lyophilized and stored at −20C. For protein analysis the lyophilized THP sample was reconstituted in urea buffer. A 100 μl sample was injected onto HPLC.

Exclusion chromatography. Samples were analyzed on an Ultimate® 3000 HPLC system using a Superdex™ 200 10/300 GL (10 × 300 mm) high performance column. The column was run at a flow rate of 1 ml per minute in 0.02 M sodium phosphate buffer (pH 6.8) containing 4 M urea. The column was heated to 40C for higher resolution. Samples were run isocratically for 30 minutes. Absorbance was measured at ultraviolet 277 nm using a VWD-3100 ultraviolet detector (Dionex, Sunnyvale, California). The THP content of urine samples was quantified as μg/ml urine by comparing them to known standard using Chromeleon®.

Sialic Acid DMB-HPLC

Lyophilized THP samples (0.5 to 1.0 mg) were used to prepare a stock solution of 1 mg/ml in Milli-Q water. For hydrolysis 100 μg samples were heated to 80C in 2 M acetic acid for 3 hours. Released sialic acids were collected by ultrafiltration through a 10,000 Da molecular weight cutoff filter and derivatized with DMB, as described by Hara et al.[16] The fluorescent DMB derivatized sialic acids were analyzed on the UltiMate 3000 system by reverse