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Immunological evaluation of urinary trypsin inhibitors in blood and urine: Role of N- & O-linked glycoproteins

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Abstract Urinary trypsin inhibitors (uTi) suppress serine proteases during inflammation. After liberation from proinhibitors (P-alpha-I and I-alpha-I) by the white blood cell (WBC) response, uTi readily pass through the kidneys into urine. A key uTi, bikunin, is attached to O-linked and N-linked glycoconjugates. Recently, uTi inhibitors, called uristatins, were found to lack the O-linked glycoconjugates. Monoclonal antibodies were produced using purified uristatin and screened for binding differences to uristatin, bikunin, P-α-I, and I-α-I. Antibody-binding patterns were characterized using immunoaffinity binding onto protein-chip surfaces and analysis by Surface Enhanced Laser Desorption/Ionization mass spectrometry (SELDI), using specimens from patients and from purified uTi standards. Antibodies were developed and used in an enzyme-linked immunosorbent assay (ELISA) method for uTi measurement in urine and plasma specimens. ELISA was performed on specimens from normal, presumed healthy, controls and from patients who had been screened for inflammation using a high sensitivity C-reactive protein (CRP) test and a complete blood count (CBC). Polyclonal antibody against uTi showed cross-reactivity with the Tamm–Horsfall protein (THP) and with proinhibitors. Screening of anti-uTi monoclonal antibodies (Mab) revealed antibodies that did not cross-react with either of the above, thus providing a tool to measure both uristatin and bikunin in urine with Mab 3G5 and in plasma with Mab 5D11. The monoclonal antibody 5D11 cross-reacts with specific N-linked glycoconjugates of uristatin present in plasma. In ca 96% of healthy adults, uTi were present at <12 mg/l in urine and <4 mg/l in plasma. We also found that patients with an inflammation and a CRP of >2.0 mg/l had higher urinary concentrations of uTi than the control population in every subject. Free uristatin and bikunin pass readily into urine and are primarily bound to heavy chains that constitute the proinhibitor form in plasma.

Keywords Uristatin · Bikunin · Urinary trypsin inhibitors · N-linked glycoproteins · O-linked chondroitin sulfate · α1-Acid glycoprotein · Tamm–Horfsfall protein · Monoclonal antibody · ELISA · Immunoassay · C-reactive protein · SELDI

Abbreviations

AMBK	α-1-Microglobulin/Bikunin precursor protein
ACT	α-1-Antichymotrypsin
AGP	Alpha-1-acid glycoprotein (orsomucoid)
ALP	Alkaline phosphatase
AMG	α-1-Microglobulin
BAPNA	N-α-Benzoyl-DL-arginine-4-nitroanilide
CRP	C-Reactive protein
CBC	Complete blood cell count
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HAT	Hypoxanthine–aminophen–thyidine

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HAS	Human serum albumin
IMDM	Iscove's modified Dulbecco's medium
Mab	Monoclonal antibody
Pab	Polyclonal antibody
P-α-I	Pre-α-inhibitor
I-α-I	Inter-α-inhibitor
PEG	Polyethylene glycol
SELDI	Surface-enhanced laser desorption/ionization mass spectrometry
THP	Tamm–Horsfall protein (uromodulin)
uTi	Urinary trypsin inhibitor
WBC	White blood cell count
ZP	Zona pellucida

Introduction

The presence of increased uTi concentrations is a significant finding in infection, acute and chronic inflammation, neoplasia and kidney diseases [1]. Bikunin is the highest molecular weight form of uTis and consists of two Kunitz inhibitor domains and two glycoconjugate chains [1, 2]. Proteases cause the release of bikunin from the interleukin-α-inhibitor (I-α-I) and the preinterleukin-α-inhibitor (P-α-I) during inflammation [3]. Bikunin is also part of the precursor protein pre-alpha-1-microglobulin/bikunin (AMBK). New forms of urinary trypsin inhibitors (e.g., uristatin) were discovered recently in patients with an acute phase reaction [4–6]. Uristatins contain either of the Kunitz inhibitor domains; however, they always lack the O-linked glycoconjugate chain. These forms were significantly more prevalent in patients' urines with the above conditions than in healthy controls. Further, they inhibited the members of the trypsin family of serine proteases. Variations owing to fragmentation of uTi and the presence in the N-linked glycoconjugate were demonstrated by Western blot analysis with an uTi polyclonal antibody (Pab) [6].

All active urinary trypsin inhibitors in a urine specimen can be measured by inhibition of trypsin [4–6]. The diagnostic use of this measurement as a nonspecific test for infection or inflammation is similar to the finding with C-reactive protein (CRP), a complete blood count (CBC) or sedimentation rate [4–10]. The first dipstick method for detecting total urinary trypsin inhibitors is based on the inhibition of a newly found enzyme substrate [4–6]. This finding permits the application of the dipsticks to the identification of urinary tract infections and kidney diseases. The strip is a urine test alternative to a blood measurement for CRP. When uTi values are $\geq$12.5 mg/l, the test is predictive of upper respiratory infections and other infections owing to bacteria or viruses as well as of inflammatory disorders.

Immunoassays for uTi offer greater clinical sensitivity in identifying disease, especially inflammation. But to date, available immunoassays are unable to distinguish among the various forms of uTi from proinhibitors (I-α-I and P-α-I) [6, 11]. The cross-reactivity of polyclonal antibodies negates tests on blood specimens where I-α-I and P-α-I are present in both healthy and infected patients. In urine, polyclonal antibodies are more effective because P-α-I or I-α-I are not typically present but still cross-react with higher molecular weight forms found during a Western blot analysis [6]. Trefz *et al*. showed that a monoclonal antibody (IATI5) produced against I-α-I also recognized P-α-I, AMBK and Bikunin [11]. Report from Kobayashi *et al*. showed monoclonal antibodies raised against AMBK were reactive to uristatin, AMBK, and bikunin [12].

Our primary goal was to produce uTi monoclonal antibodies (Mab) that did not show the cross-reactivity of polyclonal antibodies (Pab). Three monoclonal (Mab) and one polyclonal (Pab) antibodies were selected and characterized for cross-reactivity by measuring protein binding (SELDI & ELISA techniques for analysis). Protein-chip array technology, also known as "surface-enhanced laser desorption/ionization time of flight mass spectroscopy (SELDI-TOF MS)", is useful for the study of complex protein mixtures and combines affinity chromatography with mass spectrometry. This permitted us to develop ELISA methods for the assay of uristatin and bikunin in either blood or urine. A second goal was to test for correlation of uTi with high-sensitivity CRP, CBC, and the inhibition assays for uTi in hospitalized patients with or without inflammation.

Materials and methods

Urinary trypsin inhibitor standards

Standards for uTi were obtained from patients with chronic renal failure [6] (SciPac, Sittingbourne, Kent, UK, product code P205-1) (Fig. 1). Many standards were characterized by SELDI analysis as described below, and were either comprised mostly of uristatin or were mixtures of uTi, including uristatin 1 and 2 (5.9 and 8.5 kDa), uristatin (17 kDa), bikunin (~30 kDa), traces of AMBK (66 kDa), THP (~80 kDa), P-α-I (~125 kDa), and I-α-I (~240 kDa). Lot 80_117 contained 98% pure uristatin. Lot 124_111 was 80% uristatin with 10% uristatin 1 and uristatin 2 and 10% of higher molecular weight bikunin molecules. Lot 20_120 contained 20% uristatin, 50% bikunin, and 30% of high molecular weight bikunin. Standards in solutions stored for 19 weeks showed some changes at 4°C yielding larger amounts of the uristatin and smaller amounts of bikunin.

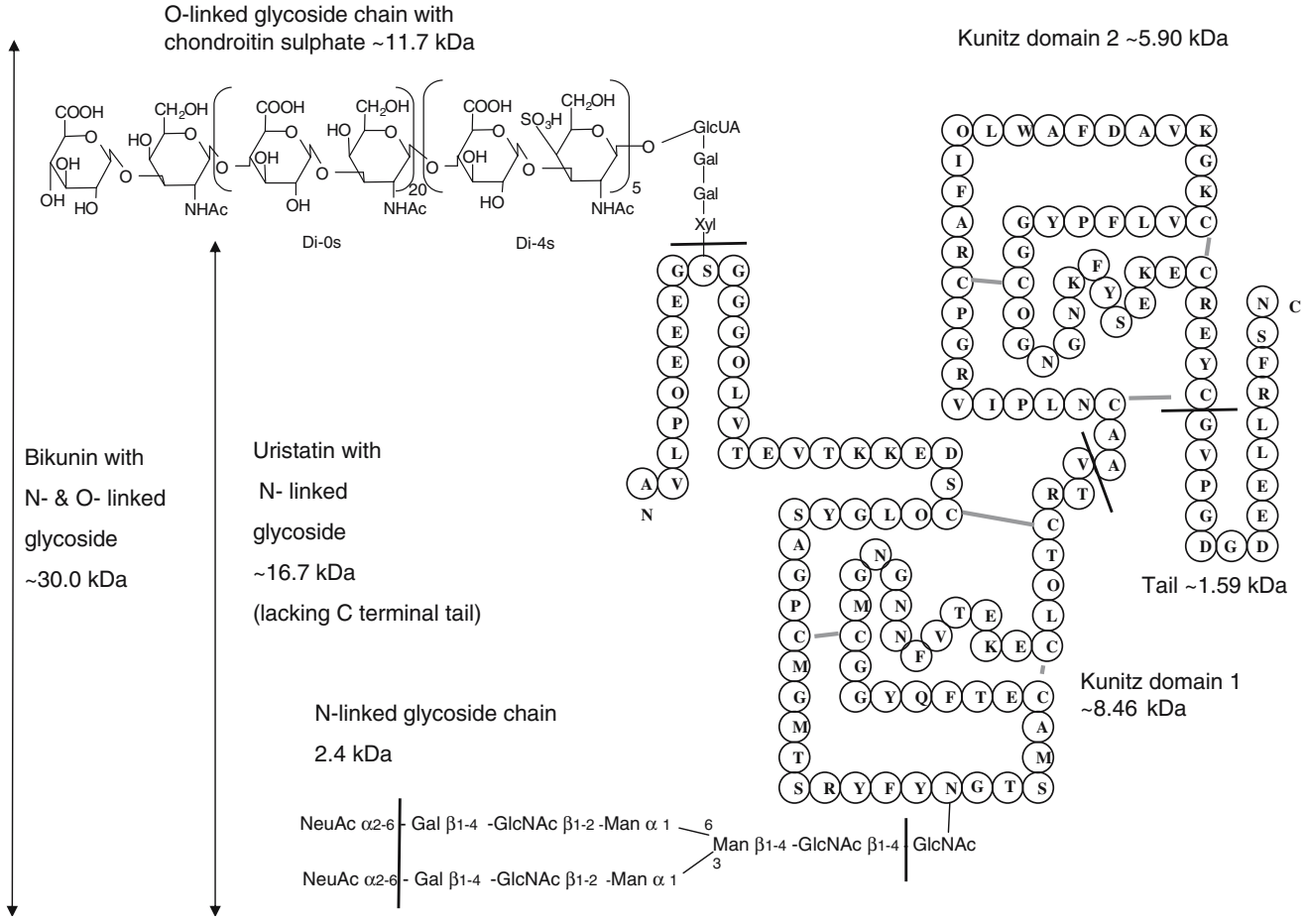

Fig. 1 Structure of bikunin and uristatin

All lots were stable at −20°C to −70°C either in solution or as lyophilized solids.

Preparation and selection of monoclonal antibodies

BALB/c mice were immunized with 100 μg of uristatin standard. After 1 month, ocular bleeds were taken, and titered by ELISA against uristatin to assess the immune response. The mice showing the best response received a booster shot of 100 μg uristatin/mouse. After 4 days, the mice were sacrificed and the spleenocytes were fused with SP2-0 Ag14 myeloma cells using PEG solution with a ratio of spleenocytes to myeloma cells of 5:1 and plated into 96-well plates using 50% PEG/HAT growth media. After 7 to 10 days of incubation at 37°C, fusion cultures were monitored for growth by feeding every 3 to 4 days with the HAT selection method followed by subculturing in HAT growth media. After 2 to 3 weeks, hybridoma colonies were tested for specific antibody production using ELISA for detection of an immune response to the uristatin. The colonies giving a positive response were transferred to 24-well plates for further expansion. The colonies testing positive were further expanded in 6-well plates in Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetal Bovine Serum (FBS) [13]. After expansion, the colonies were frozen at −70°C and then transferred to liquid nitrogen for long-term storage.

Over 50 active monoclonal antibodies from three fusions were screened after expansion; they were grouped into three groups (A, B and C) by the similarity of their binding patterns of uTi standards. The best representative antibodies from each group were selected. For the selected mother colonies, cell culture supernatants were cloned out twice using the limiting dilution method to assure that the antibodies were monoclonal. The hybridomas secreting these monoclonal antibodies in Iscove's Modified Dulbecco's Medium (IMDM) containing 30% Fetal Bovine Serum (FBS) and 10% dimethyl Sulfoxide (DMSO) were deposited at the American Type Culture Collection and designated as hybridoma ATCC 421-3G5.4C5.3B6 (Mab A), ATCC 420-5G8.1A8.5C1 (Mab B) and ATCC 420-5D11.5G8.1E4 (Mab C). Alkaline phosphatase (ALK)-labeled conjugates of monoclonal antibody were prepared for use in ELISA assays.

Characterization of monoclonal antibodies with SLEDI

Monoclonal antibodies (Mab A–C) and previously described rabbit polyclonal antibodies (Pab D) [6] were tested with uTi standards and patients' urines on chip surfaces. The binding was estimated by Surface-Enhanced Laser Desorption/Ionization (SELDI) analysis on a SELDI PBS II time of flight mass spectrometer (Ciphergen, Fremont, California) to determine the mass to charge ratios (m/z) for the proteins binding to the antibodies. Two urine specimens from patients were tested further: patient one was positive for a bacterial infection in blood and was toxemic; patient 2 was positive for a urinary tract infection by microbiological cultures [6]. Binding was measured on two types of surfaces (PS20 and RS100) using a standard incubation procedure [14]. The signal for each mass measurement was compared to the background noise to obtain the signal to noise ratios (S/N). Only masses with S/N ratios greater than 10 were accepted.

The SELDI procedure was as follows: Three microliter of 50 mmol/l $NaHCO_3$ (pH 8.0) was added to each spot on the protein chip and covered with a plate (i.e., a bioprocessor) to form sample wells followed by the addition of 1 μl antibody (1 mg/ml) to each spot and incubated at room temperature for 2 h with shaking in a controlled-humidity chamber. The solution from each spot at that time was washed twice with 5 μl of washing buffer (phosphate buffered saline (PBS) + 0.5% Triton detergent). The unbound sites were blocked with 5 μl of either 2 mg/ml BSA (bovine serum albumin) or 1 mol/l ethanolamine. After incubation at room temperature the BSA or ethanolamine was discarded and the spots were washed twice with 5 μl of washing buffer (PBS + 0.5% Triton). Five microliters of PBS was added to each spot and the chips were placed into the bioprocessor. An additional 10 μl PBS as well as 10 μl of the sample to be tested (or PBS as a control) were added to each well, followed by shaking the sealed wells at 4°C for 18 h. The wells were then washed with washing buffer and PBS and again shaken at room temperature for 2 min. The wells were rinsed twice with 300 μl of deionized water saturated with sinapinic acid; this serves as an energy-absorbing molecule during protonation of proteins bound to the antibodies. The latter are attached to the surface of the chips. The chips containing the antibody-bound specimens were analyzed for binding mass using the SELDI mass spectrometer according to the manufacturer's instructions.

Testing for cross-reactivity

The monoclonal antibodies A, B, and C as well as polyclonal antibody D raised against uTi were tested for cross-reactivity using ELISA against following glycoproteins: Tamm–Horsfall protein (THP), orsomucoid (Biomedical Technologies Stoughtan, MA), α-1-microglobulin (AMG; the Binding Site, Birmingham, UK), α-1-antichymotrypsin (ACT; Research Diagnostics, Flanders NJ), α-1-acid glycoglobulin (AGP; orosomucoid, Sigma-Aldrich, St. Louis, MO), Human Serum Albumin (HSA; Sigma-Aldrich), and two glycosidic chains: chondrotin sulfate A and B (Sigma Aldrich).

Specific cross-reactants (5 ng/well) were first immobilized on microtiter plates (PN 3690 Corning Life Sciences, Acton, MA) overnight at 4°C. Plates were then treated with SuperBlock® (Pierce, Rockford IL) to reduce nonspecific bindings after which incubated with anti-uTi antibody (0.001 to 0.1 μg/well) for another 30 min at 37°C. Alkaline phosphatase-conjugated goat anti-IgG conjugated (Sigma) was added to the wells and incubated for 30 min at 37°C. Each of the above binding steps are preceded by washing with Tris-buffered saline and 0.05% Tween-20 (TBS-Tween, Pierce). The antibody binding is visually estimated by reacting with *p*-nitrophenyl phosphate, the disodium salt (50 μl PNPP 1-Step™ solution, Pierce), and the absorbance was read at 405 nm using a spectrophotometric micro titer-plate reader (SpectroMax, Molecular Devices Corporation, Sunnyvale, CA). Proteins with any binding to antibodies A to D were assayed again as mixtures with uTi standard at 0, 20, 40, 60, 80, and 100% to determine if binding was competitive. We estimated the cross-reactant's final time at the higher urine concentrations, up to 400 mg/dl, using the final competitive ELISA assay developed for the analysis of patients' specimens (See below).

Enzyme-linked immunosorbent assays (ELISA) for clinical analysis

Patients' specimens were tested using a competitive or sandwich ELISA technique with calibrators for urine and plasma prepared in SuperBlock® blocking buffer at 0, 2.5, 5, 10, 20, 40, 60, 100 and 200 mg/l. The competitive format for Mab A–B and Pab D assay procedure is initiated by coating each well with 50 μl of 0.1 μg/ml uristatin (Lot 124-111) in TBS (Tris buffered saline). The plates were covered with adhesive tape, stored at 4°C overnight followed by washing five times with 160 μl of TBS per well (El_x 405 Auto Plate Washer, Biotek Instruments, Winooski, VT), blocked with 160 μl of SuperBlocker® per well, incubated for 30 min at 25°C (JitterBug Microplate Shaker, Beokel Scientific, Feasterville, PA), and finally washed the plates five times with 160 μl of TBS-Tween per well.

Calibrators and samples were diluted with antibody-alkaline phosphatase (ALP) conjugates in TBS using a Precision 2000 Automated Pipetting System (Biotek Instruments). The stock conjugate concentration was 0.08 μg/ml for Mab A and B and 0.40 μg/ml for Pab D. Analysis of

urine required a 100-fold dilution of ALPMab$_{(A\&B)}$ and 1,000-fold dilution for ALP-Pab$_{(D)}$. A 1,000-fold dilution of the Mab was used for plasma assays. Diluted calibrator or specimen in 50 µl volumes were loaded into the coated wells, incubated at 25°C for two hours, and washed five times with 160 µl TBS-Tween per well. The sandwich format was used for Mab C by coating each well with 80 µl of 10 µg/ml of Mab C in TBS. Plates were covered with adhesive, stored at 4°C overnight, washed five times with 160 µl of TBS per well, blocked with 160 µl of SuperBlocker® per well, incubated for 30 min at 25°C, and washed five times with 160 µl of TBS-Tween per well. The calibrators and samples were diluted 1,000-fold with SuperBlocker for both urine and plasma assays. After dilution, 50 µl of calibrator or specimen were loaded into the coated wells, incubated at 25°C for 2 h, washed five times with 160 µl TBS-Tween per wells, and then 50 µl of 0.40 µg/ml ALP-Mab$_{(D)}$ (Pab D) conjugate was loaded into each well. The plate was incubated at 25°C for another 2 h followed by a second TBS-Tween wash cycle.

The antibody binding is estimated by incubation at 25°C for 30 m (JitterBug Shaker) after addition of PNPP (50 µl/well; 1-Step™ solution, Pierce), which is hydrolyzed by bound ALP conjugate, followed by addition of 25 µl 2 M NaOH to each well to stop the reaction. The plates were read at 405 nm on a FLx 800 microplate Reader (Biotek Instruments). The average absorbances and SD were calculated for the triplicate results. Unknowns were determined from standard curve after a sigmoidal nonlinear curve-fitting routine (variable slope) using the Prism 4 program (GraphPad Software). We analyzed each calibrator and specimen in triplicate using 96-well plates. Separate plates were used for each antibody method. Triplicates were spaced on the microplates as widely as possible for best assay precision The CV was between 2.4 to 14.8% for all the calibration steps.

Specimen collection from patients and controls

We obtained urine and whole blood specimens from 188 hospitalized patients. Clean-catch midstream urines were obtained from all patients. The blood specimens were collected into EDTA-containing tubes (BD Vacutainer®) and immediately separated them into plasma and cells. Specimens were stored at 4°C until tested. If not tested within 4 h, storage was at −20°C. Inflammation was found in only 7 of 188 patients with a high sensitivity CRP result of ≥2.0 mg/l (BN II nephelometer Dade Behring, Deerfield IL). The control group of 181 patients without inflammation had an average CRP of 0.4 ± 0.5 mg/l and an average white blood cell (WBC) count of 6,800 ± 2,400 cell/µl (Cell-Dyne 4000, Abbott Diagnostics, Santa Clara, CA). There was only one result above 12,400 cell/µl , the upper limit of abnormal leukocyte counts. The affected group (n = 7) with a CRP of ≥2.0 mg/l had only a slightly elevated WBC of 9,100 ± 2,900 cell/µl).

Inhibition assays for uTi

Inhibition assays using an arginine peptide substrate (BAPNA) and trypsin on a CX3 analyzer (Beckman) allowed the uTi to be determined in urine according to the previously published Cobas Fara II method [12]. Calibration using uTi standards at 0 (undetectable) and at <2.5, 6.25, 12.5, 25, 50, 75, and 100 mg/l permitted the assay calculation from a linear regression line. The precision of the Beckman analyzer (duplicates on five days) was 5.17 ± 0.39 mg/l (SD) and 48.4 ± 0.58 mg/l (SD). The nominal or "labeled" concentrations of the controls from the manufacturer were 6.25 mg/l and 50 mg/l.

We also performed a uTi dipstick test on the Clinitek® 50 strip reader (Bayer HealthCare) to obtain uristatin concentrations of 0 (undetectable at <6.25 mg/l), 12.5, 25, and 50 mg/l [6] .The strip measured uTi controls with values of 0 and 12.5 mg/l correctly 99% of the time for five determination/day over ten days.

Results

Monoclonal antibody screening

Monoclonal antibodies were classified into three groups (A, B and C) based on their response to three lots of purified uTi standards (Fig. 2). Group A antibodies had the strongest

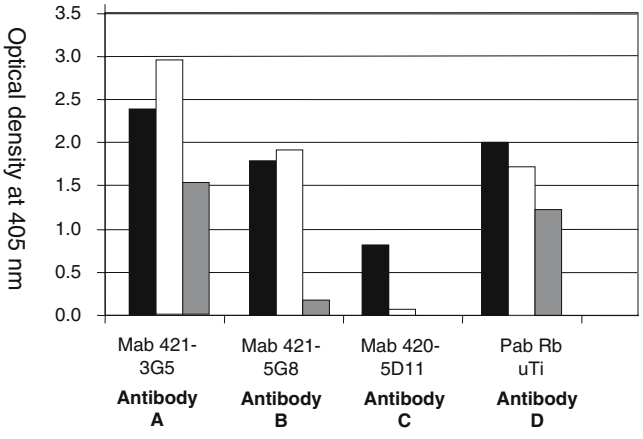

Fig. 2 Antibody binding to urinary trypsin inhibitors standards. Shown are the typical binding patterns for monoclonal antibodies (A, B, C) and polyclonal antibody (D) to three lots of purified uTi standards; Lot 20_120 (*solid bars*) contains 20% uristatin, 50% bikunin, and 30% of high molecular weights; Lot 124_111 (*open bars*) was 80% uristatin with 10% uristatin-1 and 2, and 10% of bikunin with higher molecular weight fragments present; and lot 80_117 (*gray bars*) contained substantially only uristatin (98% pure)

binding affinity to uTi lot number 124_111 over other lots. The reduced concentration of high molecular weight proteins in lot 124_111 suggested these clones would be less reactive to P-α-I and I-α-I. Previously, Pab was found to be more reactive to these proinhibitory forms [6]. We selected clone 421-3G5 for further study as it had the strongest binding affinity of the group. Group B antibodies had equal binding affinity to lots 124_111 and 20_120. We chose clone 421_5G8 as it had the lowest binding affinity to lot 80_117. The group C antibody had a strong binding affinity for standards that contained a significant amount of proteins with molecular weights of 60 to 80 kDa. This is consistent with earlier findings with western blot using patients' clinical urines that showed Group C clone 420_5D11 had bound strongly to protein in the 60 to 80 KDa range.

Surface-enhanced laser desorption/ionization (SELDI)

The SELDI results for the three Mab and the Pab are given in Table 1. The values represent the *m*/*z* (mass number) of proteins bound to the antibodies after reaction with the uTi standards and the urine of two patients with bacterial infections. This method determined the molecular weights of the bound forms and does not rely on any interpretation of the composition of the standard. The high sensitivity of the SELDI method demonstrates antibody binding to many more forms than are detectable by comparison to standards. Those binding events of high affinity, frequency, or importance are indicated in italic bold numbers as determined by signal-to-noise ratios of binding events (Table 1). The secondary binding events are bold while weak binding events are in plain text.

As seen in Table 1, SELDI and uTi standards demonstrated that the primary binding for Mab A was strongest for the small uristatin 1 or 2 fragments, uristatin, and AMBK. All four antibodies bound to all of the above proteins, with the uristatins showing the strongest binding. We found no significant proinhibitor binding at the low concentrations found in the standards derived from urine. The primary binding for Mab B was with uristatin 1 or 2, less so for uristatin, no binding of bikunin, and weak proinhibitor binding. The primary binding for Mab C was strongest for THP, uristatin, and uristatin 1 or 2, and weaker for bikunin or AMBK. The polyclonal binding occurred with all forms including the proinhibitors and THP. All Mab exhibited strongest binding to uristatin 1 or 2 with molecular weights of 2.8, 3.5, and 3.9 kDa. The SELDI results also show several low affinity binding sites, but these were not strong enough to affect specificity.

The results in Table 1 using two patients' urines demonstrate the same primary binding patterns for anti-

Table 1 Analysis of antibodies A–D using SELDI mass spectrometry after immunoaffinity binding to urinary trypsin inhibitors

Expected mass for antibody	Mass (kDa) of antigen bound to antibody[a,b]			
	Antibody A	Antibody B	Antibody C	Antibody D
	Mab 421-3G5	Mab 421-5G8	Mab 420-5D11	Polyclonal Ab (anti-uristatin)
Uristatin 1 or 2. Average molecular weights of 5.9 and 8.5 kDa with kDa range 2–12	***2.82***, ***3.41***, ***3.48***, ***5.38***, ***5.57***, **6.30**, **7.24**, 3.5, 3.8, 4.0, 5.6, 6.0, 6.3, 8.0, 9.2, **9.8, 10.9**	***2.82***, ***3.41***, ***3.48***, 4.0, 5.4, **7.2**, **9.18, 9.85**	***2.82***, **3.0**, **3.5**, **4.7**, 3.7, 5.4, 5.6, 7.2, 3.7, 10.8	**2.8**, ***5.0***, **5.4**, **7.2**, **10.7**, 3.5, 4.0, 5.9, 6.0, 6.3, 8.1, 10.7
Uristatin. 17 kDa, range 11–22	***11.8***, **18.0**, 16.3, 13.4, 14.0	12.0, 14.0, 13.5, **21.1**	***11.9***, 13.5, 21.1,	**13.4**, **14.0**, 16.3
Bikunin. 30 kDa, range 21–46	***21.1***, 33.6, 35.2, 33.5, 45.9	23.0, 22.7	23.3, 41.6	**21.2**, **22.6**, **23.3**, ***33.4***, 33.1, 35.2, 42.3
AMBK. 66 kDa, range 42–70	***67.0***	No significant peaks	No significant peaks	58.6
THP. 85 kDa, 80–91 kDa	81.9, 91.1	No significant peaks	***80.2, 80.6, 81.9***	82.6, 79.2
p-alpha-I. 125 kDa	No significant peaks	128.5, 132.1	No significant peaks	**101.1**, **103.7**, **106.3**, 120.0, 123.4, 133.0, 142.7
I-alpha-I. 240 kDa	No significant peaks	No significant peaks	No significant peaks	No significant peaks

[a] Antibody binding observed with two patients' urine and the uTi standard. One patient was positive for a blood bacterial infection (sepsis) and the other was positive for a bacteriuria (urinary tract infection) as determined by microbiological cultures.
[b] Masses (kDa) rounded to nearest 0.1 kDa. Peaks with highest S/N relative to other peaks are reported in bold italic face, followed by bold face, and then plain text for lowest S/N binding events.
S/N Signal-to-noise ratio.

bodies A to D shown with the uTi standards. The SELDI results, however, also demonstrated several new forms of uristatin 1, uristatin 2, uristatin (containing both 1 & 2), and bikunin within the expected molecular weight ranges. In particular, primary binding to 2.8, 5.4, 7.2, 11.8, and 21.1 kDa proteins was observed in patients' specimens but not in the purified standards. Greater variations in the pathological conditions or the pooling and purification of standard to the target molecular weight could explain the difference. The SELDI binding also showed cross-reactivity to THP by the antibodies C and D in both the standards and patients' urines.

Cross-reactivity testing

Cross-reactivity testing of the three monoclonal antibodies as determined by ELISA using uTi, Tamm–Horsfall protein, and α1-acid glycoprotein is shown in Figs. 3, 4 and 5 and is in agreement with the findings by SELDI. All three Mabs bound preferentially to uTi with A and B showing better binding affinity than Mab C as seen in Fig. 3. No cross-reactivity of Mabs A or B was observed to either Tamm–Horsfall protein (THP) or α-1-acid glycoprotein (AGP) while Mab C cross-reacted specifically to THP (Fig. 4) and AGP (Fig. 5). However the binding affinity of Mab C was ~30 times stronger for uTi than AGP. None of the monoclonal antibodies showed any specificity toward α-1-microglobulin (AMG) of the precursor protein AMBK, α-1-antichymotrypsin (ACT), human serum albumin (HSA), or chondroitin sulfate A and B. Pab were about 50% cross-reactive to THP and AGP (data not shown).

Competitive and ELISA methods when used for patients' specimens showed no cross reactivity to Mab A and B, with HSA (400 mg/dl), THP (4 g/l), AGM (400 μg/ml), AGP (1 g/l) or ACT (400 μg/ml). Mab C remained cross reactive to THP (at (50 mg/l) and weakly cross reactive to AGP (at 2 g/l). Pab cross-reacted with THP at ≥100 mg/l. This is the region of trace proteinuria.

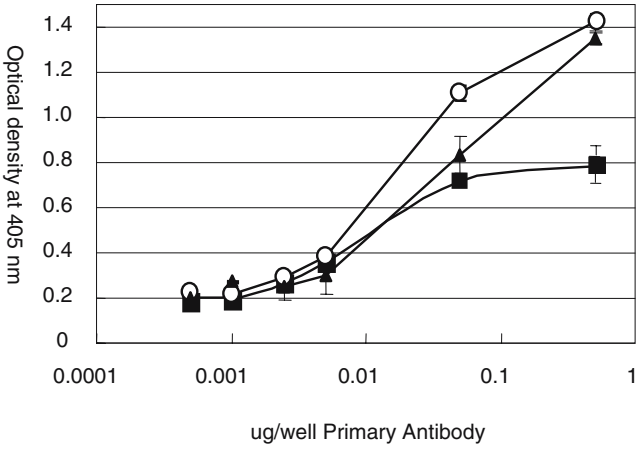

Fig. 3 Monoclonal antibodies A, B, and C reactivity to uristatin. Concentration of Mab A (*circles*), Mab B (*triangles*), and Mab C (*squares*) plotted *versus* the observed binding to uristatin (5 ng/well). Optical density at 405 nm reported after reaction of p-nitrophenyl phosphate (PNPP) with ALP

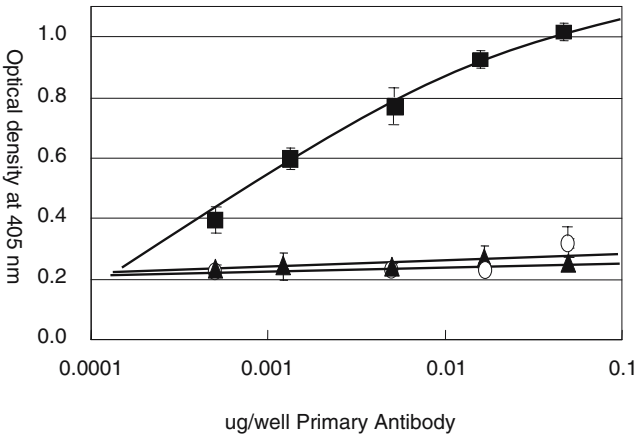

Fig. 4 Monoclonal antibodies A, B, and C reactivity to Tamm–Horsfall protein. Concentration of Mab A (*circles*), Mab B (*triangles*) and Mab C (*squares*) plotted *versus* the binding observed to Tamm–Horsfall Protein (5 ng/well). Optical density at 405 nm reported after reaction of PNPP with ALP

Testing of normal and affected patients

Normal patients could be distinguished from those with inflammation with a CRP cutoff value of (<2.0 mg/l). Patients in the normal group (n = 181) had an average uTi by the quantitative inhibition assay of 8.8 ± 6.9 mg/l while the population with inflammation (n = 7) had an average uTi of 38.8 ± 6.0 mg/l. This agrees with previously reported cutoffs of 12 mg/l for those with inflammation [4–6]. The strip inhibition assay was negative for 153 of the normal group and positive for three of the affected group. The quantitative Beckman assay was negative for 150 of the normal group and positive for six of the affected group and

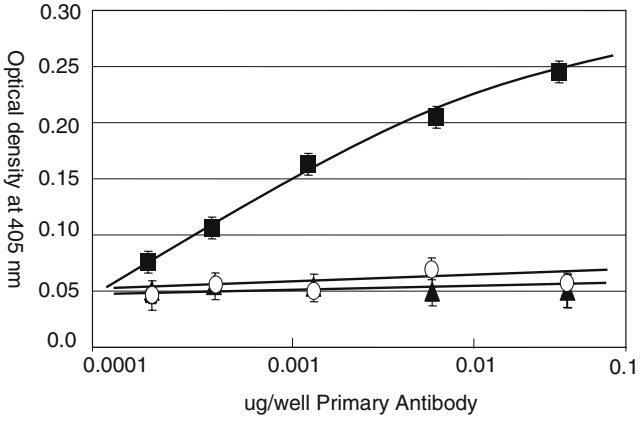

Fig. 5 Monoclonal antibodies A, B, and C reactivity to alpha-1-glycoprotein. Concentration of Mab A (*circles*), Mab B (*triangles*) and Mab C (*squares*) plotted *versus* the binding observed to alpha-1-glycoprotein (50 ng/well). Optical density at 405 nm reported after the reaction of PMPP with ALP

agrees with the difference in detection limits previously reported [5, 6]. The quantitative inhibition assay is more accurate at the lower range and is more predictive of a CRP of 2.0 mg/l than the strips. The strip assay is more predictive of CRP values of ≥10 mg/ml [5].

Inhibition assays cannot be performed on blood owing to their nonspecific nature with any trypsin like enzyme or inhibitor in blood affecting the rate. Mab A and B and Pab were predictive of negative results by inhibition of urine assays. A positive Mab C was not predictive as expected by cross reactivity in urine to THP (false positives = 94). The negatives were as expected by the quantitative and strip assay. Inhibition assay positives showed better agreement with the immunoassay results for Mab A–B. The best correlation was with Mab A and the inhibition methods; here, specimens analyzed by the antibody assay were positive >80% of the time by the inhibition assays. We did not find false positives owing to exogenous inhibition in the immunoassay method.

Antibody tests were superior in separating those with and without inflammation as compared to the enzyme inhibition method (Table 2). Clinical assays of uTi in urine were best made with Mab A which detected all of the affected group and were negative in 167 of the normal group (Table 2). The normal population had an average uTi of 3.6 ± 5.4 mg/l; the affected population had an average uTi of 28.0 ± 7.0 mg/l ($p < 0.001$). Mab B and Pab were also predictive of six patients in the affected group and negative in 159 of the 164 of the normal group (Table 1). A cutoff of 7.8 mg/l gave the best separation of normal and affected patients. In practical use, Pab cannot be used because of the cross reactivity of THP causes many false positives. Proteinuria was absent from the patients and controls in this study (negative by protein dipstick). Mab C with 100% cross reactivity to THP was not useable for the urine tests. Only 81 of the control group were negative and just three of the seven in the affected group were detected.

The reference range for uTi in urine was similar for Mab A, B, and Pab. More than 96% of the normal group had a uTi value of less than 12 mg/l. The normal urinary range observed for Mab C was much higher with a 40 mg/l cutoff required to include 96% of the normal group. THP is normally present in urine with a reference range of up to about 50 mg/l.

Blood concentrations of uTi measured by Mab A or B were not significantly different between the normal and affected populations (Table 1). uTi values in whole blood were much higher than the concentrations found in urine suggesting cross-reactivity with proinhibitors. We confirmed this by western blot analysis. Using Mab A and B to assign a reference range for proinhibitors in blood, we found that 96% of the normals were 25 to 150 mg/l (average

Table 2 Agreement of ELISA procedure (for antibodies A–D) and the high sensitivity CRP method, urine specimens

Urine specimens							
Primary proteins measured	Antibody method	Healthy controls (n = 181)			Patients with inflammation (n = 7)		
		Mean (mg/l)	SD (mg/l)	Controls correctly identified[a,b]	Mean (mg/l)	SD (mg/l)	Patients correctly identified[b,c]
uTi	Mab A	3.6	5.4	93%	24.9	34.9	100%
uTi	Mab B	4.1	6.1	88%	30.9	46.8	88%
THP	Mab C	12.6	13.1	NS	18.9	21.6	NS
uTi	Pab	3.6	5.6	91%	21.1	27.2	88%
Plasma specimens							
Primary proteins measured	Antibody method	Controls (n = 181)			Patients with inflammation (n = 7)		
		Mean (mg/l)	SD (mg/l)	Controls correctly identified[b]	Mean (mg/l)	SD (mg/l)	Controls correctly identified[b]
Proinhibitor	Mab A	76.4	37.6	NS	112.0	45.8	NS
Proinhibitor	Mab B	85.3	28.4	NS	99.7	13.7	NS
uTi	Mab C	1.0	1.1	NS	0.7	0.4	NS
Proinhibitor	Pab	26.4	16.9	NS	24.3	9.6	NS

[a] The percentage of patients (n = 181) with hsCRP < 2.0 mg/l and urinary trypsin inhibitor < 7.8 mg/l.
[b] NS ($P > 0.001$) was observed between patients with and without hsCRP at 2.0 mg/l.
[c] The percentage of patients (n = 7) with hsCRP ≥ 2.0 mg/l and urinary trypsin inhibitor ≥ 7.8 mg/l.
NS no statistically significant difference.

value was ~80 mg/l). Blood values for uTi measured by Pab were also higher than the concentrations found in urine and no difference in the blood test results were found between patients and controls.

Blood uTi measurements could be made with Mab C and showed the free uTi to be 1 to 2% of the total proinhibitor concentration. We determined that a blood uTi test was not a predictor of general inflammation as measured by CRP. Using Mab C to assign reference values for blood free uTi (e.g., uristatin and bikunin), a cutoff of 4 mg/l was above 96% of the normal group. Comparison of blood and urine measurements supports the notion that free uTi passes readily through the glomeruli and that, in blood, these proinhibitor proteins are normally bound.

Discussion

The SELDI analysis using uTi antibodies demonstrated that variations in uTi were far more common than previously reported [6]. Other groups have reported the insensitivity of SDS-PAGE analysis as compared to SELDI during studies of urine proteins in urolithiasis [14]. The variation of uTi was far greater in individual patient's specimens than expected with purified standards for whole uristatin and bikunin. The between-patient variation in uTi would be expected owing to the fragmentation of peptide structure by serine proteases released in patients with inflammation. Modification of the O-linked proteoglycan chain during inflammation has also been reported [15–17], and modifications of the complex N-linked glycan would be expected.

Uristatin fragments of uTi, previously identified, lacked the proteoglycan chain but were inhibitory toward serine proteases [6, 17–19]. These smaller uTi fragments of Kunitz domain either lacking or containing the N-linked glycan chain are commonly found in urine. Strong binding of the Mab A and B to these low molecular weight forms of uTi makes these ideal assays for urines where these small fragments are likely the best representation of the epitopes bound by each antibody. As these fragments are also common to I-α-I and P-α-I, the assay of blood with Mab A or B measures these proinhibitors proteins as well as the smaller fragments. These proinhibitors do not readily pass into urine, and cross-reactivity is not a problem in urine testing, because the concentration for proinhibitors is lower [6].

The cross-reactivity of Mab C and Pab with THP and AGP is not due to common amino acid sequences since there is less than 10% homology [20]. THP has EGF-like light chains binding and ZP domains [21–25] while AGP has a lipocalin domain for binding certain steroid hormones, basic drug, IgG, heparin and vanilloid [26, 27]. Bikunin has two Kunitz domains for serine protease inhibition and no domains common to THP or AGP (Fig. 1). Bikunin, uristatin, THP, and AGP all have a complex N-linked glycan in common that varies in the degree of fucosylation, sialylation, and branching [17–19].

The N-linked glycan of uTi has a biantennary structure (Fig. 1) [17–19]. The number of glycoforms of uTi is unknown. Tamm–Horsfall protein has five N-linked glycan chains that can be bi-, tri-, and tetra-antennary complexes [28–30]. The number of glycoforms of THP has also not been determined although it is known to be highly donor dependent. AGP has five N-linked glycan chains that can be bi-, tri-, and tetra-antennary complexes with 40 known glycoforms of which the biantennary complexes are rare [31–34].

The greater abundance of uTi forms now reported suggests a far greater possibility for a diagnostic role for fragments and their associated N-linked glycan chain in pathological conditions. Mab C appears to be ideal for measuring uTi fragments in blood through binding to the biantennary N-linked glycan of uTi. While this N-linked glycan is common in THP, the latter is only excreted in the proximal tubules of the kidney and is not found in serum [35]. In urine, cross-reactivity to THP is a concern. The weak cross reactivity to AGP does not appear to be a significant factor in blood. Even though AGP is present in substantial amounts in blood (ca 1 g/l), the biantennary species is an uncommon glycoform.

Glycoconjugates of the cross-reactive proteins (THP and AGP) play a role in the body's defense mechanisms. The N-glycan of THP has hemagglutination properties. The influenza virus binds to sialic acid through the neuaminidase-type glycoprotein on the virus cell membrane [35, 36]. THP also has one O-linked proteoglycan chain involved and is membrane-bound [28, 29]. One N-linked mannose chain has *E. Coli* binding properties [37]. AGP is an acute-phase protein with membrane binding properties to modulate monocyte, granulocyte, and lymphocyte binding to the endothelial cells [26, 31]. The immunomodulatory activity of AGP is dependent on glycosylation with formation of sialyl Lewis X and leading to endothelial cell adhesion through P- and E-selectins thereby blocking immunocell adhesion [27]. The sialyl Lewis X is known to bind to the influenza virus [35]. Expression of sialyl Lewis X and bi-tetra-antennary complexes of AGP is known to increase early on with inflammation [26, 27] while greater fucosylation occurs with diabetes [34]. The role of the N-glycan of uTi is not known, although several acute phase processes could be implicated.

Conclusion

New methods now allow the direct measurement of specific forms of uTi with monoclonal antibodies in urine and

blood. This is the first report of monoclonal antibodies that do not show an interfering cross reactivity to proinhibitors in blood and THP in urine. The antibodies have also been shown to serve as tools for uncovering the role of uTi and glycoconjugation with either ELISA or SELDI methods. To date it has not been shown whether certain glycoforms or uTi fragments occur more frequently in chronic inflammation, are more easily distinguished in acute inflammation, are more common in certain tissues, or are formed by certain pathogens. It is reasonable to assume that certain forms of uTi can result from chronic disease, and these new tools will help in identifying individuals with inflammation. This work should advance the biochemical understanding of inflammation.

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