Urinary β_2-Microglobulin Is Associated With Acute Renal Allograft Rejection

William S. Oetting, PhD, Tyson B. Rogers, MS, Thomas P. Krick, MS, Arthur J. Matas, MD, and Hassan N. Ibrahim, MD, MS

• ***Background:*** **Identifying urinary biomarkers associated with acute rejection (AR) of kidney allografts could improve recipient care by allowing AR to be diagnosed noninvasively and treated earlier. We attempted to identify novel biomarkers associated with AR by analyzing urinary proteins by using matrix-associated laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS).** ***Methods:*** **Using MALDI-TOF MS, we analyzed urine samples from 30 renal allograft recipients with biopsy-proven AR, 15 allograft recipients without AR, preoperative samples from 29 kidney donors, and 10 subjects with proteinuric native kidney disease.** ***Results:*** **In samples obtained at the time of AR, we identified a protein peak at 11.7 kd that correlated strongly with AR. In regard to its predictive power for AR, this protein peak showed sensitivity of 83.3%, specificity of 80%, positive predictive value of 89%, and negative predictive value of 70.6%, suggesting that this protein is highly associated with AR. We identified this peak as being β_2-microglobulin. This was validated by using enzyme-linked immunosorbent assay, which documented the presence of high urinary β_2-microglobulin levels in subjects with AR.** ***Conclusion:*** **β_2-Microglobulin could be a strong biomarker for AR if used in conjunction with other biomarkers, producing an AR-specific urinary protein signature. This possibility must be confirmed in a larger cohort of kidney transplant recipients.** ***Am J Kidney Dis*** **47:898-904.**

INDEX WORDS: Acute rejection; proteomics; biomarkers.

EARLY DIAGNOSIS AND prompt treatment of acute rejection (AR) episodes in kidney allograft recipients are important, potentially minimizing graft functional deterioration and the rate of graft loss.[1,2] Currently, serum creatinine (sCr) levels are used as the major biomarker for evaluating kidney function. However, because sCr levels may increase for numerous reasons other than AR and AR treatment is associated with complications, sCr levels are used only as a guide for further evaluation.[3,4]

Percutaneous renal allograft biopsy is the gold standard for AR diagnosis. Kidney recipients with a 25% or greater increase in sCr levels generally undergo biopsy, which is associated with significant costs and potential complications.[5-7] A more specific biomarker (or set of biomarkers) for AR potentially could decrease the need for biopsy and may permit earlier AR diagnosis. Identifying AR-specific protein biomarkers would allow for rapid, noninvasive, and economical testing. Ideally, such biomarkers should be highly sensitive and specific to differentiate AR from other forms of graft dysfunction. It also would be highly advantageous if such biomarkers could predict long-term transplant outcome. For kidney transplant recipients, urine would be the most logical source of an AR-associated biomarker.

The introduction of new mass spectrometry (MS)-based technologies has allowed identification of novel biomarkers. Matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF MS) and the related technique, surface-enhanced laser desorption ionization MS, provide means for analyzing a complex mixture of proteins in solution. These methods allow samples to be analyzed in a cost-effective (compared with biopsy) and timely manner. Several studies used these technologies to analyze urine protein profiles in kidney recipients.[8-10] In those studies, differences were identified in the protein patterns observed; some differences could be associated with AR. Clarke et al[10] found 2 protein peaks

From the Departments of Medicine, Biochemistry, Molecular Biology and Biophysics, and Surgery, University of Minnesota, Minneapolis, MN.

Received December 7, 2005; accepted in revised form January 30, 2006.

Originally published online as doi:10.1053/j.ajkd.2006.01.034 on April 6, 2006.

Support: Funded in part by grant M01-RR00400 from the National Institutes of Health to the General Clinic Research Center at the University of Minnesota. Potential conflicts of interest: None.

Address reprint requests to Hassan N. Ibrahim, MD, MS, University of Minnesota, Department of Medicine, 420 Delaware St SE, MMC 736, Minneapolis, MN 55455. E-mail: ibrah007@umn.edu

0272-6386/06/4705-0021$32.00/0

doi:10.1053/j.ajkd.2006.01.034

associated with AR: those biomarkers showed sensitivity of 83% and specificity of 100% in regard to their possible identification of AR. Conversely, Schaub et al[8] found a more complex pattern consisting of many proteins: 94% of kidney recipients with AR (versus 18% without clinical or histological evidence of AR and 0% of healthy controls) had this AR-specific pattern.

In an effort to identify novel protein biomarkers associated with AR, we used a nonbiased method for protein detection. We analyzed urine samples from kidney recipients with and without AR by using MALDI-TOF MS and compared them with healthy controls and patients with proteinuric native kidney disease.

METHODS

We collected spot urine samples, with informed consent, from 4 groups of individuals: 15 consecutive kidney transplant recipients with normal kidney function, 30 consecutive kidney transplant recipients with biopsy-proven AR in the first 6 months posttransplantation (n = 30), 10 individuals with native kidney disease that was clinically diagnosed or biopsy proven, and 29 kidney donors as healthy controls. Mean sCr level was 5.8 ± 2.6 mg/dL (512 ± 227 μmol/L) in subjects with native kidney disease. All kidney recipients received antibody induction therapy. Maintenance immunosuppression was with calcineurin inhibitors and either mycophenolate mofetil or sirolimus. We collected 50 mL of urine from each individual, then centrifuged 2- to 15-mL tubes of urine at 2,100*g* to remove cellular debris. Samples were stored in 1-mL aliquots at −80°C.

Initially, we analyzed samples by means of MALDI-TOF MS. We identified protein peaks associated with AR by comparing protein spectra of urine from transplant recipients with AR with urine from transplant recipients without AR and kidney donors. Proteins of interest were isolated by using gel electrophoresis and identified by using MALDI-TOF MS/MS. To confirm protein identification, we used iodoacetamide analysis, which determines the number of cystines in a polypeptide.

MALDI-TOF Analysis

For MALDI-TOF MS analysis, samples were thawed and 30 μL of urine was acidified with 0.5 μL of 10% trifluoroacetic acid (TFA) per 10 μL of sample. Protein then was subjected to ZipTip (C4; Millipore Corp, Billerica, MA) processing according to the manufacturer's instructions. This step both concentrated the protein and removed salts that would interfere with MS analysis. The ZipTip initially was wet with 2 × 10 μL of a solution of water:acetonitrile:TFA (50:50:0.1) and washed 2 × 10 μL with a solution of water:TFA (100:0.1). Acidified urine was drawn up into the ZipTip and expelled slowly 10 times, then the tip was washed 5 times with 10 μL of 0.1% TFA in water. Proteins were eluted by washing the ZipTip with 1.7 μL of water:acetonitrile:TFA (75:25:0.1) by slowing drawing the solution up and down through the tip 10 times. To this, 1.5 μL of water:acetonitrile:TFA (50:50:0.1) saturated with sinapinic acid was added, and the 1.5 μL was spotted onto the target. After drying, the sample was subjected to MALDI-TOF MS analysis in a Bruker Biflex III mass spectrometer equipped with a nitrogen laser (Bruker Daltronics, Billerica, MA). Each assay consisted of a combined signal from 500 laser pulses. At least 10 different areas of the target were analyzed for each assay.

Gel Electrophoresis

Protein from 1 mL of urine was precipitated with 4 volumes of acetone at −80°C. Proteins were resolved by using a 10% sodium dodecyl sulfate-polyacrylamide gel, stained with Deep Purple Total Protein Stain (Amersham Biosciences, Piscataway, NJ), and visualized on the Typhoon 8600 (Amersham Biosciences). Protein-stained bands were aseptically excised from the gels manually and subjected to tryptic digestion[11] on the Genomic Solution ProPrep (Department of Biochemistry, Molecular Biology and Biophysics Proteomic Analysis Core facility).

MALDI-TOF MS/MS Protein Identification

To identify the protein in the gel band, we used MALDI-TOF MS/MS analysis. We subjected gel slices to 2 series of dehydration and hydration steps by adding, incubating, and removing acetonitrile, followed by adding, incubating, and removing 25 mmol/L of NH_4HCO_3. We then subjected the gel slices to reduction in the presence of 10 mmol/L of dithiothreitol (DTT)/25 mmol/L of NH_4HCO_3 at 60°C for 30 minutes. The DTT solution was aspirated, and 55 mmol/L of iodacetamide/25 mmol/L of NH_4HCO_3 was added for 30 minutes at 25°C. The iodacetamide solution was aspirated, followed by 2 series of dehydration and hydration steps as described. Next, we subjected the gel slices to tryptic digestion with 12 ng/μL of trypsin (Promega, Madison, WI) in 25 mmol/L of NH_4HCO_3 and 5 mmol/L of $CaCl_2$ at 37°C for 10 hours. The reaction was stopped by adding formic acid to a final concentration of 0.1% and transferred to a 1.5 siliconized Eppendorf tube. The gel slice was extracted with acetonitrile for 10 minutes, followed by the addition of 0.1% formic acid for 10 minutes. Solutions from a single plug were pooled, frozen, and concentrated in a speed vacuum. Samples then were rehydrated in buffer for liquid chromatography/MS. Peptides were separated by means of capillary high-performance liquid chromatography using an acetonitrile gradient and analyzed by nanoelectrospray quadrupole-TOF MS/MS (QSTAR Pulsar *i* mass spectrometer; Applied Biosystems Inc, Foster City, CA). Fragments then were identified using ProID software (Applied Biosystems).

Iodoacetamide Analysis

The number of cysteines in a protein can be determined with DTT and iodoacetamide treatment, allowing for confirmation of the identity of the protein. For each cysteine in the protein, the mass will increase by 57 d, which can be measured by using MALDI-TOF MS. In our study, we made urine samples containing the 11.7-kd protein alkaline by using sodium bicarbonate. We then added DTT (0.1 mol/L), incubated at 37°C for 10 minutes, and then added iodoacet-