Immunossay of Urine of a Specific Marker for Proximal Tubular S3 Segment

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Urinary intestinal alkaline phosphatase (EC 3.1.3.1; IAP) is a marker of the S3 segment of the human kidney proximal tubule. An accurate enzyme–antigen immunossay (EAIA) with a high-affinity specific monoclonal antibody (IAP250) developed for this marker has a detection limit below the lowest IAP activity found in urine samples of normal subjects. The intra- and interassay CVs were <5%. Mean analytical recovery of pure IAP added to urine was 102% (SD 6%), and the EAIA results correlated well with immunoreactivity (measured by a sandwich ELISA), suggesting that the EAIA detected all of the IAP in urine. In healthy individuals (ages 20–80 years) the IAP concentrations, expressed as urinary creatinine ratios, ranged from 0.1 to 2.0 U/g (5–95 percentiles) without major differences related to sex and age. Workers exposed to mercury, which affects the S3 segment, showed an increased IAP elimination; abusers of analgesics, which affect more distal parts of the nephron, did not. As opposed to currently measured markers, the EAIA offers easy, accurate, and precise measurement of early alterations in the S3 segment.

Additional Keyphrases: enzyme–antigen immunoassay • enzyme-linked immunoabsorbent assay • monoclonal antibody • alkaline phosphatase • isoenzymes

For many years an active search has been under way for proteins or other molecules for which appearance or concentration change in urine indicates alterations of cell integrity, in particular of nephron segments. Differences in the distribution of these markers along the nephron are considered to be useful in identifying the specific anatomical site of nephrotoxin-induced damage. Indeed, drugs and other chemical substances, e.g., analgesics (1), mercury (2), cisplatin (2), aminoalcohols (3), and cyclosporine (4), exert their toxic effects at different positions along the nephron.

However, the use of urinary markers in assessing renal injury has several limitations. Much of the disparity and paucity of reliable results in this area are from insufficient segment specificity of the current markers and imprecise assay techniques (5). Moreover, the detection of urinary protein excretion or enzyme activity can be influenced by various factors that are independent of cell integrity, i.e., urinary pH, osmolality, and the presence of inhibitors and activators. This is particularly well documented for β2-microglobulin, necessitating oral bicarbonate loading some hours before its determination in the urine to prevent acid-dependent β2-microglobulin degradation in the urine (6). Finally, almost no studies have correlated abnormal enzymuria with verified functional or histological evidence of tubular damage (7).

The use of monoclonal antibodies directed against particular antigens localized along the nephron can lead to the identification of unique renal markers that are specific for different anatomical sites in the nephron and that can be detected reproducibly by immunossay techniques (8). Using a monoclonal antibody (IAP250) to human intestinal alkaline phosphatase (IAP), we demonstrated (9) that, in the normal human kidney, IAP is exclusively located at the brush border of tubuleepithelial cells present in the S3 segment of the proximal tubules, from which it can be released into urine. Here we describe an accurate and precise assay of this marker.

Materials and Methods

Reagents and Apparatus

p-Nitrophenyl phosphate was purchased from Janssen Chimica (Beerse, Belgium). Trypsin (type III; 11.250 kU/g), poly-L-lysine, and 3,3',5,5'-tetramethylbenzidine were from Sigma Chemical Co. (St. Louis, MO). Rabbit antiserum raised against human placental AP but cross-reacting with IAP, biotinylated rabbit anti-mouse Ig antiserum, avidin, biotinylated horseradish peroxidase, and rabbit anti-mouse Ig serum were from Dakopatts (Glostrup, Denmark). Casein and 2-amino-2-methyl-1-propanol as well as all other reagents ("pro analysis") were from Merck (Darmstadt, F.R.G.). Flat-bottomed Micro-immunoplates I were from Nunc (Roskilde, Denmark). The DU-8 spectrophotometer was from Beckman (Brea, CA) and the EAR400 multimwell reader was from SLT-Labelinstruments (Grödig, Austria). A Histokinette 2000 was from Reichert Jung GmbH (Nussloch, F.R.G.).

Urine Samples and Subjects

Untimed urine samples were collected in stabilizing buffer (9) (50 mL/L urine) provided by N.V. Innogenetics (Ghent, Belgium) and stored at 4 °C. Before the assay, insoluble material was removed by spontaneous sedimentation in the standing samples. To study the corre-
lation between IAP activities obtained by enzyme-antigen immunoassay (EAIA) and by sandwich enzyme-linked immunosorbent assay (ELISA), we collected 130 untimed urine specimens from healthy individuals and from renal-transplant patients receiving cyclosporine treatment (10).

To establish normal reference values, we investigated the distribution of urinary IAP activities in relation to age and sex for subjects recruited from two large-scale epidemiological studies. The first group was taken from a project on early indicators of nephrotoxicity in workers due to environmental pollutants (1989–1990; 72 women and 311 men). The second consisted of 37 men and 89 women, selected from control subjects for a longitudinal prospective study on the role of analgesics abuse in the development of chronic renal failure (1984–1991). To complete the range of ages in both sexes, we collected urine samples from women younger than 30 years (n = 17) and in men older than 60 years (n = 16).

Two toxin-exposed groups were analyzed. Analgesics abusers (n = 160) were studied as a nonrisk group, because this group is primarily affected in the renal medulla. On the other hand, mercury-exposed workers (n = 104) were chosen as a risk group manifesting changes of the S3 segment, because mercury particularly affects this segment. The analgesics abusers were selected from the general population along with their age- and sex-matched controls (n = 160). Mercury-exposed workers were recruited in three chloralkali factories, including greatly exposed workers from Banská Bystrica (Czechoslovakia), and their controls (n = 83) in other factories. On the basis of questionnaire data, potential control subjects exposed to nephrotoxic products (i.e., lead, cadmium, mercury, and organic solvents) were excluded.

Subjects with hypertension (blood pressure >160/100 mmHg, with or without antihypertensive treatment) and those with a positive Combur® dipstick test for glucose were excluded.

Thirty-five patients (20 women and 15 men, ages 18–75 years) with various degrees of proteinuria (300–5000 mg/L) were followed in the outpatient Nephrology Clinic (University Hospital Antwerp, Belgium). IAP in serum was determined from total AP activity and from the isoenzyme pattern obtained by agarose-gel electrophoresis (11). Urinary IAP was measured as described above.

Methods

**EAIA:** To measure IAP activity in urine, we further developed a previously described EAIA system (9). Briefly, microtiter plate strips (12 × 8) were coated with anti-IAP antibody IAP250 and washed three times with phosphate-buffered saline (PBS) containing, per liter, 0.5 mL of Tween 20 (PBS/Tween 20) and 0.25 mol of glycine. After drying the plates overnight at room temperature, we stored them at 4°C in a container with silica gel as the desiccant.

To each well of a microtiter plate strip, we added 100 μL of assay diluent (PBS containing, per liter, 1 g of casein, 1 mmol of MgCl₂, and 50 μmol of ZnCl₂) followed by duplicate 100-μL portions of urine or of IAP standards. After incubating the wells overnight at room temperature, we washed them three times with PBS/Tween 20. We added 200 μL of substrate solution to each well and measured absorption at 405 nm after 30 min of incubation at room temperature and after 1 and 2 h of incubation at 37°C.

**Sandwich ELISA:** As an alternative, we developed a sandwich ELISA, based on IAP250, for measuring IAP antigen concentrations in urine (9). Briefly, after we coated microtiter plate wells with rabbit antiserum against human placental AP and that cross-reacts with IAP, we added assay diluent and standards or urine samples. The sample volume and incubation time were the same as in the EAIA protocol. After the overnight incubation, IAP250 was added for 3 h at 37°C, followed by incubation with biotinylated rabbit anti-mouse Ig antibodies and with preformed avidin–biotinylated horseradish peroxidase complex. After reaction with tetramethylbenzidine and 0.6 mL of H₂O₂ per liter of citrate-phosphate buffer, pH 4.3, we stopped the reaction after 30 min by adding 50 μL of 4 mol/L H₂SO₄ and measured absorbance at 450 nm. Results for the IAP standards were expressed in units of AP activity.

**IAP standards:** Human dimeric fetal IAP (140 kDa) was purified from meconium as described elsewhere (9). The preparation had a specific activity of 2900 kU/g and a final purity of >95%, as evidenced by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (12), and was stored in liquid nitrogen as a stock solution (300 U/L) in 20-μL aliquots in assay diluent. From this solution, standards ranging from 0.10 to 10.00 U/L were prepared by serial dilution in the assay diluent before each assay.

One unit of activity (1 U) was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of p-nitrophenyl phosphate per minute at 30°C. AP activity was assayed at pH 10.4 by use of substrate solution containing, per liter, 16 mmol of p-nitrophenyl phosphate, 350 mmol of 2-amino-2-methyl-1-propanol, 2 mmol of magnesium acetate, 1 mmol of ZnSO₄, and 2 mmol of hydroxyl-EDTA according to the recommendations of the International Federation of Clinical Chemistry (13).

**Normalization of urinary IAP values:** Bias from not using timed urine samples was overcome by representing the results as IAP activity (U/L) per urinary creatinine concentration (mg/L) and expressing the ratios as U/g.° The urinary creatinine concentration was measured by the modified Jaffé reaction (14).

**Localization of IAP in normal human renal tissue:** We performed immunohistochemical staining for IAP as described previously (9, 15). Briefly, we cut 1.5-mm-thick slices of renal tissue from the surface of the kidney to the papilla, fixed them for 1.5 h in buffered 40 mL/L formaldehyde, and embedded them in low-melting-point paraffin (16). We mounted 4-μm-thick sections on poly-L-lysine-coated slides and treated them with trypsin.

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7 To convert to U/mol, divide by 0.00884.
Binding of the monoclonal antibody IAP250 was revealed by an indirect avidin–biotin–peroxidase method with use of 3-amino-9-ethyl carbazole and H₂O₂ as substrates. The sections were counterstained with methyl green and mounted in glycerine gelatin.

Results

Assay Validation

Increasing amounts of stabilizing buffer added to urine progressively enhanced the recovery of urinary IAP by 20% (SD 4%; n = 10) during subsequent EAIA measurements, reaching a plateau with the addition of 50 μL of buffer per 1 mL of urine. The presence of stabilizing buffer had no influence on urinary creatinine determinations. The pH of stabilized urine samples varied between 6.0 and 7.5. The IAP activity in sediments encountered in ~10% of stabilized urine samples was below the detection limit after Triton X-100 extraction. Therefore, enzyme assays could be performed on urine supernates.

Analysis of the fractional binding of the IAP in standards and in urine samples as a function of time confirmed that equilibrium in the EAIA was reached more rapidly at 37 °C than at room temperature (Figure 1). At 37 °C, equilibrium was reached after 6 h (3 t₁/₂, equivalent to 87.5% of the amount bound at equilibrium) as opposed to after ~10 h at room temperature. The long equilibration times necessitated including an overnight incubation step in the protocol, which resulted in complete binding of IAP in the standard and in the urine samples (n = 20).

Urinary IAP, having a smaller mass (83 kDa) than the IAP in the standard (140 kDa) (9), reached equilibrium slightly faster (Figure 1).

Linear calibration curves could be constructed (r >0.999) for bound IAP activity (A₁₄₀₀) vs added IAP at amounts between 0.00 and 10.00 U/L when plates were read after 30 min of incubation at room temperature. When incubated at 37 °C and read after 1 or 2 h, calibration curves were linear between 0.00 and 2.50 U/L.

The detection limit of 0.02 U/L in the EAIA, calculated as the mean and 3 SD for 12 replicates of the zero standard, was well below the lowest urine IAP activities encountered in a normal population (not shown). The intra-assay CV (10 parallel measurements of four urine samples with different IAP concentrations) was ~4%. The interassay CV (a urine sample stored at 4 °C assayed on 10 consecutive days) was ~5%.

To investigate whether the urine matrix exerted any effect on the accuracy of urinary IAP determinations, we tested 10 different urine samples undiluted and diluted two- and fourfold. Suppression of the matrix effect was achieved by diluting the urine samples twofold. Likewise, when a known amount of IAP standard (0.5, 1.0, or 2.0 U/L) was added to urine samples (n = 26), the recovery (102%, SD 6%) confirmed that matrix effects were eliminated in twofold-diluted urine samples.

The stabilities of the antibody-coated microtiter plates and of the IAP in standard solutions and in stabilized urine samples were investigated at different temperatures (~80 °C, ~30 °C, 4 °C, room temperature, and 37 °C) and as a function of time (0, 5, 11, and 22 days; 2 and 6 months). The immunoplates and the IAP standards were stable for at least six months at 4 °C. No loss of urinary IAP activity in a stabilized urine sample was observed after storage for three weeks at 4 or ~80 °C (Figure 2). For storage times up to six months, 4 °C was preferred over ~20 °C. A part of the activity loss during storage at ~20 °C could be explained by the effect of a freezing–thawing cycle. We experimentally verified this in seven stabilized urine samples frozen at ~20 °C and thawed immediately; mean activity decrease was 8% (SD 9%). Small variations in pH in stabilized urine samples (pH 6.0–7.5) had no influence on the stability of urinary IAP activities.

To investigate potential interferences from osmolality in urine, various NaCl or urea concentrations (0–500 mmol/L) were added to four different urine samples. IAP activity was not significantly modified by these additives.

Correlation between EAIA and ELISA

We assayed 130 urine samples from healthy individuals and renal-transplant patients by both EAIA and
ELISA. Regression analysis demonstrated an excellent correlation (Figure 3).

Values of Urinary IAP

Although no major differences were noticed between the distribution of the urinary IAP activity/creatinine ratios in women and men according to age (Figure 4), cutoff values (P95) for women were slightly greater. In both groups the median (P50) remained stable over the different age categories.

IAP/creatinine indices were repeatedly analyzed in urine samples of healthy individuals (n = 8) for five consecutive days (three samples per day) (Figure 5). For some urine samples, diurnal fluctuations of IAP/creatinine ratios did not exceed the upper cutoff zone (17).

In 12 of 35 proteinuric patients, urinary IAP was increased. In only 2 of these 12 subjects was IAP increased in serum. In 7 of the 35 proteinuric patients, IAP in serum was >10 U/L (96th percentile; 18), and IAP in urine was increased in only 2 of the 7.

Localization of IAP in Normal Human Kidney

Positive immunohistochemical staining for IAP was present only on the brush border of proximal tubule cells in the outer stripe of the outer medulla and in the medullary rays (Figure 6A). According to the topological distribution of these IAP-containing tubular cross-sections in the kidney (medullary rays and outer stripe of outer medulla), and in view of the well-developed brush border in these cells, we concluded that IAP was present exclusively in the S3 segment of the proximal tubule, according to the criteria of the International Society of Nephrology (19) (Figure 6B).

Epidemiological Data

Figure 7 demonstrates that IAP activities were greater in mercury-exposed workers (n = 104) than in the controls (n = 83; Mann–Whitney U test: P < 0.001). Mercury exposure was measured in urine, and median (range) values were 89.3 nmol/g (25.4–896.7 nmol/g) and 6.5 nmol/g (1.0–23.5 nmol/g) for exposed workers and control subjects, respectively. IAP activities in an-

Discussion

Before urinary enzymes are used as indicators of renal damage or alterations, the following should be established: enzyme characterization, molecular configuration, cellular localization and segment specificity, an
easy and precise assay system, and stability. Here we show that IAP has these prerequisites for being an ideal marker.

The human kidney contains two antigenically distinct AP isoenzymes, tissue-nonspecific AP and IAP (20), both contributing to AP activity in urine (20–22). IAP is exclusively in the brush border of the S3 cells of the proximal tubule, whereas tissue-nonspecific AP is located throughout the entire proximal tubule (9). This contrasts with many other urinary markers (e.g., N-acetylglucosaminidase, which is found throughout the proximal tubule as well as in renal papillae and glomeruli; 23).

Despite its lower molecular mass (85 kDa) (9), urinary IAP retains almost all its biochemical and immunological characteristics; its origin is most likely renal. The extracellular luminal-site localization of the ectoenzyme IAP in the S3 cells makes its release into the tubular fluid obvious. IAP in serum, with its 140-kDa molecular mass, cannot be filtered through the glomeruli of the normal kidney (24). Our findings in proteinuric patients also suggest that urinary IAP does not originate from serum.

Total AP has often been analyzed in urine (25), but the measurement of its activity is complicated by interfering material (26). AP activity can only be determined after inhibitors (e.g., pyrophosphates) are removed by dialysis. Drugs and drug metabolites (e.g., nitrofurantoin and ethionamide; 26) also interfere with AP determination. In our EIA, the immunological recognition of IAP is not hampered by the urinary matrix in urine samples diluted twofold, nor is IAP activity measurement affected by inhibitors or activators.

The distribution of IAP activities in urine samples from normal subjects shows the assay to be sufficiently sensitive to accurately quantify IAP, and the analytical recoveries are complete. Because urinary IAP concentrations are extremely low, i.e., in nanomoles per liter, overnight incubation is necessary for complete equilibrium to be reached.

Both the enzyme activity and the immunoreactivity of urinary IAP are stable in buffered urine samples (pH >6.0) stored at 4 °C for at least three weeks. Optimal long-term preservation is at −80 °C. This contrasts with the stability of β₂-microglobulin, which can be degraded even before voiding (9) because of the acidity in the urinary bladder. Buffering of freshly voided urine is adequate for IAP measurement.

Recent results in workers exposed to mercury, a well-known tubular toxin interfering preferentially with the S3 segment of the proximal tubule, showed that IAP was increased in these workers compared with control subjects (27). In contrast, analgesics abusers did not show increased IAP activities compared with age- and sex-matched control subjects, which agrees with the fact that analgesics does primarily involve the renal papillae (28). The median IAP values in analgesics abusers and
their controls are somewhat higher than those for the mercury-exposed workers because the former are mostly older women and the latter are mostly younger men.

In conclusion, by using a high-affinity monoclonal antibody (9) with a high specificity for active IAP (specific activity of 3 MU/g; I2), we developed a simple, reproducible, and highly sensitive EIAA. IAP in urine is a promising marker of alterations in the S3 segment of the human proximal tubule.

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References