DeCREASED TAMM-HORSFALL PROTEIN IN LITHIASIC PATIENTS

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OBJECTIVES: Tamm-Horsfall glycoprotein (THP) is the most abundant substance of renal origin appearing in urine. It seems to be one of the major inhibitors of calcium oxalate crystal nucleation and/or aggregation, but its role in the pathogenesis of stone formation has not yet been clarified. The present work was undertaken to quantify THP excretion in lithiasic (L) patients, so as to determine if these levels are different from those of control subjects (C).

DESIGN AND METHODS: THP was isolated from human urine by precipitation steps, rabbit antiTHP antibody was obtained and its specificity determined, an ELISA was developed and technical conditions standardized, and quantitative measurements of urinary THP were performed on samples from L patients, who had suffered more than one lithiasic episode, and from C subjects. Microtiter plates coated with THP or diluted urine samples were subjected to successive incubation with antiTHP and alkaline phosphatase antirabbit IgG.

RESULTS: A good correlation between measured absorbance and THP concentration in standard and urine samples was observed. Data, expressed as Median and Interquartile Range, are 388/209-626 μg THP/mmol creatinine for C (n = 85) and 124/82-171 μg THP/mmol creatinine for L (n = 23).

CONCLUSIONS: We have obtained an antiserum antiTHP that can be used in the ELISA technique to determine reliable urinary THP values. The results show a significant decrease of THP excretion in recurrent stone formers compared to controls (p < 0.001) and may have interesting implications in the pathogenesis of urolithiasis.

KEY WORDS: Tamm-Horsfall glycoprotein; urolithiasis; stone formers; uromodulin; ELISA; lithiasic patients.

INTRODUCTION

Tamm-Horsfall glycoprotein (THP) is the most abundant protein of renal origin in normal urine (1) and a major constituent of urinary casts (2,3). It has been immunohistochemically localized in cells of the lining of the ascending limb of the loop of Henle and of the distal convoluted tubules in mammalian kidneys (4,5). Although THP's physiological function still remains unclear, it was first described as an inhibitor of viral-induced hemagglutination (6), and it seems that this protein may also play a role in regulating the activity of several circulating cytokines (7,8). Evidence is available to support a role of this protein in urothelial defense against infection because it has been demonstrated that Escherichia coli with type I fimbriae can be trapped by THP (9), being bacteria-coated by this glycoprotein and less susceptible to phagocytosis by polymorphonuclear lymphocytes than uncoated bacteria (10,11).

In relation to nephrolithiasis, THP has been shown to be present in the core of calcium oxalate renal stones (12); however, it is not clear whether it is a passive support or an active participant in stone formation. Moreover, results of in vitro assays developed with the aim of elucidating the role of THP on stone production are controversial. Some authors have described THP as an inhibitor (13), or even as a promoter (14) of stone formation, and others have claimed that it has no effect (15). More recent reports have postulated that THP and nephrocalcin are two major inhibitors of calcium oxalate crystal aggregation in normal urine, and presented evidence to demonstrate that stone formers excrete defective aggregation inhibitors (16,17). Taking into account those observations, it has been hypothesized that, not only defective THP, but a decreased THP excretion may facilitate the development of renal stones. Thus, the aim of the present work is to quantify urinary THP in lithiasic patients, so as to determine if these levels are lower than those of control individuals. To measure THP urinary levels, an enzyme linked immunosorbent assay (ELISA) was developed, employing previously isolated urinary THP and antibody antiTHP raised in rabbits.

METHODS

PREPARATION OF THP FROM HUMAN URINE

Isolation

THP was isolated from 6 liters of urine obtained from healthy adult donors according to Tamm and
Protein analysis

Freeze-dried material was reconstituted in 0.2 mol/L phosphate buffer pH 7.4 containing 6 mol/L urea. Protein concentration was determined according to Bradford (18).

Discontinuous polyacrylamide gel electrophoresis was performed according to Laemmli (19). Briefly, 20% sodium dodecyl sulphate (SDS) solution was added to the samples up to a 5:1 SDS:protein ratio (w/w), together with 0.1% (w/v) Bromophenol Blue tracking dye, and after heating (100 °C, 2 min), volumes of 10–25 μL of each sample were placed on a 1 mm thick gel layer formed by a stacking gel (T: 4%; 125 mmol/L Tris-HCl; 0.1% (w/v) SDS; pH: 6.8) and a running gel (T: 10%; 375 mmol/L Tris-HCl; 0.1% (w/v) SDS; pH: 8.8). The buffer in the electrode reservoirs was 25 mmol/L Tris; 192 mmol/L glycerine; 0.1% (w/v) SDS, pH: 8.3. Proteins were detected by staining with either 0.1 g/L Coomassie Blue R 250 or Silver Staining (Sigma Chemical Co., Saint Louis, MO, USA). Standard commercial THP (BT382 THPGlycoprotein, 95%, Biomedical Technologies, Inc., Stoughton, USA) and molecular weight markers (Sigma Chemical Co.) were employed to determine standard migration distances.

Production of antiTHP-antiserum

Two 6 month-old male white rabbits of approximately 600 g of body weight each, were immunized with 1 mg of THP preparation in a complete Freunds adjuvant (Sigma Chemical Co.). This injection was repeated every 10 days (with incomplete Freunds adjuvant) for 6 months. Blood samples were obtained from the ear vein at different times during the treatment, to determine the antiserum titer by the Ouchterlony technique (20). When the desired antiserum titer (1:128) was obtained, the animal was sacrificed, and pool bleeds were kept in aliquots at −20 °C until needed.

Control of antibody specificity

The antiserum specificity was determined by Western blot following the procedure described by Towbin et al. (21). Immediately after SDS-PAGE, proteins were transferred to a nitrocellulose (NC) membrane (0.2 μm pore size, Sigma Chemical Co) using a buffer of 25 mmol/L Tris, 192 mmol/L glycine; 20% (v/v) methanol. After the blotting step, the NC blots were soaked for 30 min at 25 °C in a NaC1 solution with 5% powdered milk (Molico, Nestlé, Switzerland) and washed 3 times with borate buffer (20 mmol/L boric acid; 150 mmol/L NaC1; 0.05% (v/v) Tween 20). The NC membranes were then rinsed with TBS and incubated overnight with the appropriate dilution of either experimental or commercial THP-antisera at 4 °C. After rinsing with TBS, a horseradish peroxidase conjugated antirabbit IgG antibody was employed as second antibody. The peroxidase activity was revealed by immersing the NC paper in a freshly prepared solution of 1.9 mmol/L 4-chloro-1-naphthol in 50 mmol/L Tris-HCl buffer pH 7.4, containing 0.2 mol/L NaC1 and 0.01% hydrogen peroxide. Proteins gave blue positive bands.

Previously, dot blot assays were performed to determine the adequate concentrations of antigens and antibodies.

ELISA procedure

An ELISA methodology was developed following the one described by Reinhart et al. (22).

THP stock solutions (1 mg/mL) were prepared by dissolving freeze-dried THP or standard THP (Biomedical Technologies INCBT 382 THGlucoprotein) in Buffer A (15 mmol/L Na2CO3, 25 mmol/L NaHCO3, 0.02% (w/v) sodium azide, pH 9.6).

Six samples of serial dilutions (between 1:8,000 and 1:128,000) were applied as 200 μL aliquots to the ELISA microplates (Corning R, New York, USA). Urine samples were diluted 1:800 in Buffer A and different dilutions were applied to the plates. Assays were run 4 times for each dilution of either samples or standards. Controls containing only Buffer A were run simultaneously.

After overnight incubation at 4 °C, the plates were washed 4 times with buffer PBS-Tween pH 7.4, containing 137 mmol/L NaCl, 147 mmol/mL KH2PO4, 8.1 mmol/L Na2HPO4, 2.68 mmol/L KCl, 0.02% (w/v) sodium azide and 0.05% (v/v) Tween. Then, 0.1% (w/v) bovine serum albumin (Sigma Chemical Co.) was used as blocking agent to prevent nonspecific adherence to the plates. After 1 h incubation at room temperature, the plates were washed 4 times with PBS-Tween. Experimental antiTHP antibody solution was diluted 1:256 in PBS-Tween, whereas the commercial antibody (Biomedical Technologies BT-590) was diluted 1:2,000 in the same buffer. Volumes of 200 μL of either antibody solution were added to each well and left to stand for 4 h at 37 °C. After 4 washings with PBS-Tween, the second antibody:alkaline phosphatase conjugated mouse antirabbit
IgG (D487, Dako, Copenhagen, Denmark) was added in a dilution of 1:2,000. After overnight incubation at room temperature, the plates were washed 4 times with PBS-Tween. To develop the color, 5.6 mmol/L sodium p-nitrophenylphosphate in a buffer containing 10% (v/v) diethanolamine and 0.5% (w/v) magnesium chloride, pH 9.6, was added to the plates, and after 20–25 min, the color reaction was stopped by the addition of 50 μL of 3 mol/L NaOH to each well. Absorbance readings were performed immediately afterward at 405 nm in ELISA reading equipment (Microwell system, Organon Teknika, Belgium).

A standard curve was established for each plate. The urinary THP concentration was determined using multiple dilutions of the same sample.

INDIVIDUALS

Inclusion criterion for the study was a history of at least 2 well-documented episodes of renal calculi within the previous 4 years. Exclusion criteria were severe chronic disease (diabetes mellitus, chronic heart failure, or cancer), treatment with diuretics, and renal failure (serum creatinine above 133 μmol/L). Using these criteria, 23 patients were included in the current study. These patients were recurrent calcium stone formers without dietary restrictions and, by the time of the study, they were not taking medication for their stone disease and they had been given instructions to increase their fluid intake. The mean age of the 15 men and 8 women who took part in the study was 45 years. The mean age and range for men were 50/30–74 years and for women, 40/32–45 years. Stone studies revealed that calculi were formed by calcium oxalate monohydrate, except in 2 cases; 1 was made of uric acid and the other of a mixture of calcium oxalate and carbonate.

Control subjects were hospitalized patients and laboratory staff without a history of stone disease who were under no medical treatment. Of the 85 total controls, 39 were men and 46 were women. The mean age in the control group was within the range of the patients’ ages (41/25–60 years for men and 42/30–55 years for women).

Urinary samples

First-void urine samples were obtained from patients and control subjects and immediately stored at -20 °C until assay. Creatinine and calcium concentrations were determined by an autoanalyzer (Hitachi 704, Boehringer-Mannheim Diagnostics, Indianapolis, USA).

STATISTICAL ANALYSIS

Results are expressed as Median and Interquartile Range, the latter including values between the 25th and 75th percentiles. The Kolmogorov-Smirnov 2-sample test was employed to determine differences between groups.

Results

THP preparation

A total of 150 mg of native THP was obtained from 6 L of human urine by repeated salt precipitation. The final preparation, dialyzed and freeze-dried, appeared as a single band of approximately M, 80,000 after SDS-PAGE and was clearly revealed on both gel and Western blots. The electrophoretic migration rate was similar to that of the standard human THP, the experimental THP comigrated with the commercial one. No additional bands were detected on either Coomassie Brilliant Blue or Silver staining.

Crossed assays between experimental and standard THP and antiTHP upon Western blot demonstrated specificity of the rabbit antiserum. Both antibodies identified a single protein, whether they reacted with the urinary THP obtained in the laboratory or with the commercial standard protein. The optimum concentration was determined to be 1:128 for experimental antiserum and 1:32,000 for the commercial antibody.

ELISA

Serial dilutions, either of experimental THP or of the standard preparations, correlated well with absorbance readings over a wide range of concentrations; a linear response was obtained between 0.1 and 0.8 absorbance units. Calibration curves from absorbance against THP concentration using commercial THP and antiTHP were in good agreement with those observed when employing experimental antibody. Slope and intercept calculated from those graphs varied from plate to plate. So, standard THP concentration curves were run in each plate.

The intra-assay variability of the THP assay was 4.1% and the interassay variability, determined in aliquots of the same sample on different days, was 4.4%.

Individual day-to-day variations, determined by testing samples of 5 controls collected on different days, were found to be between 10 and 20%.

THP was added to urine, increasing the urinary THP concentration by 10 and 20 mg/L. The recovery rates were 84% and 101%, respectively. The sensitivity of the assay was in the range of 8–10 μg THP/L.

Urinary THP determination

Data of THP concentration expressed as creatinine concentration rates in first-void urine samples were in good correlation to the same rates determined in 24-h urine samples.

The THP excretion in the control population (n = 85) was 388/209–626 μg THP/mmol creatinine, with a range value between 37 and 1,100 μg THP/mmol creatinine. The same parameters for the lithiasic patients (n = 23) were 124/82–171 μg THP/mmol cre-
atinine. These values were included in a range from 24 to 343 µg THP/mmol creatinine (Figure 1). No differences of THP excretions were found between men and women, either in the C or L groups. The results show a significant decrease of THP excretion in recurrent stone formers compared to controls ($p < 0.001$).

**Discussion**

There are still no appropriate markers to predict or follow the development of lithiasis. Moreover, the exact role of the THP in urine of normal people and stone formers is not yet well defined. However, several indications that THP could be related to growth and/or aggregation of calcium oxalate crystals raised interest in measuring the amount of THP excreted by lithiasic patients.

With this aim, we developed an ELISA for urinary THP based on the method described by Reinhart et al. (22). The coincident electrophoretic mobilities between the urinary isolated protein and the THP standard allowed us to characterize the experimental THP.

The antiserum raised in rabbits proved to be effective in dilutions of up to 1:256 and this concentration was satisfactory for our assay. The specificity of the THP antibody was further established by the reaction with both the experimental and the standard THP. Absorbance values obtained with different dilutions of the urinary isolated THP showed good parallelism with THP standard curves after the reaction with either the experimental or the standard antiserum. This result confirms the specificity of the obtained antiTHP antiserum that proved to be useful in the development of an ELISA technique to determine reliable urinary THP values.

The individual variability (10–20%) found in the present study was lower than other reported values, 42.7% (22) and 41% (23). We suppose that it may be due to having been determined in only 5 individuals. On the other hand, intra- and intervariability and sensitivity of the assay were in close agreement with previous reported values (22,23).

We analyzed urine samples from 85 controls and 23 patients with a history of recurrent calcium oxalate stone formation. The results show that the urinary excretion of THP is markedly decreased in lithiasic patients (Figure 1). Nevertheless, they do not allow us to differentiate between the low THP excretion due to depressed synthesis or to decreased cellular secretion. The urinary THP levels show no correlation with the number of lithiasic episodes or with age or sex inside each group.

Several other reports have determined the TH glycoprotein daily excretion in different groups of healthy adults. Some reported mean values were 64.22 ± 35.12 mg THP/L (24), 62.1 ± 51.9 mg THP/L (25), 70 ± 26 mg THP/24 h (26), and a range from 20 to 80 mg THP/g creatinine corresponding to the mean ± 2 SD (27). The results in the present work cannot be strictly compared to those previously published by others because of the different forms of data expression, but estimations, made on the basis of normal amounts of creatinine excretion, show good agreement between the information in those works and in the present one.

It has been established that recurrence of stone formation is a major problem in up to 50% of patients who form calcium stones (28). The results here presented support the usefulness of the urinary THP measurements and allow us to suggest that THP may have interesting implications in the pathogenesis of urolithiasis.

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**References**


