Analysis of an Expanded Width of Albumin Fraction by Cellulose Acetate Membrane Electrophoresis in IgA Nephropathy Urine Before Treatment

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Cellulose acetate membrane electrophoresis with colloidal silver stain revealed that the width of the albumin fraction in IgA nephropathy (IgAN) urine before treatment was significantly expanded. This phenomenon was not shown in IgAN urine after treatment or in non-IgAN urine. There was a reverse correlation between the width of the albumin fraction and the albumin concentration in IgAN urine. By immunofixation, Tamm-Horsfall protein (THP) was located in the same position as the albumin band in IgAN urine before treatment; however, in the urine of a healthy subject it was located in the same position as α_1 -globulin. By ELISA,

the THP-albumin complex concentration in IgAN urine before treatment was significantly higher than in the other two diseases. The width of the albumin fraction and the sodium ion concentration of the urine were significantly correlated. The THP/albumin ratio in IgAN urine before treatment was significantly higher than in the other two groups. This suggests that the characteristic expanded width of albumin found by immunofixation indicates a THP-albumin complex, and that the sodium concentration of urine is involved in the formation of this complex. *J. Clin. Lab. Anal.* 17:37–43, 2003. © 2003 Wiley-Liss, Inc.

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INTRODUCTION

IgA nephropathy (IgAN) is the most common glomerulonephritis in the world (1). About 20–30% of IgAN patients develop progressive renal failure within 10–20 years from the onset of disease (1–3). The Ministry of Health and Welfare, and the Japanese Kidney Society established the diagnostic criteria of IgAN in Japan, as follows: macroscopic hematuria, proteinuria, high serum IgA level, and the predominant presence of IgA deposits in the glomerular mesangium. At present, renal biopsy is used to differentiate IgAN from other glomerulonephritis. However, renal biopsy is invasive, and results are affected by the accuracy of the biopsy position; therefore, it is necessary to determine a useful diagnostic marker in the urine to replace renal biopsy. In 1992, Hotta (4) reported that the analysis of the urinary macrophage phenotype is useful for

evaluating renal injury. Although this is an effective method, the procedure is complicated. As a simpler method, we attempted to detect the specific urinary protein fraction in IgAN before treatment using cellulose acetate membrane electrophoresis with colloidal silver staining. With the use of this method, which we described previously (5), we could detect even very small amounts of Bence Jones protein (BJP) without concentrating the urine. As a result, we could identify

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the specific protein fraction in IgAN urine before treatment.

MATERIALS AND METHODS

Reagents

The cellulose acetate membranes used in electrophoresis were purchased from Fuji Photo Film Co. (Tokyo, Japan). A Titan gel silver stain kit was purchased from Helena Laboratories (Beaumont, TX). Antisera against albumin were purchased from DAKO (Glostrup, Denmark). Sheep anti-human uromucoid IgG and POD-conjugated sheep anti-human uromucoid IgG were from The Binding Site (Birmingham, UK). Other chemical reagents were purchased from Wako Pure Industrial Chemicals (Osaka, Japan).

Subjects

All of the patients in this study were monitored in the Department of Nephrology, Sendai Shakaihoken Hospital. Urine samples were obtained from 46 patients (20 men and 26 women) with IgAN, who had been identified by renal biopsy specimens. Of these samples, 18 were obtained before tonsillectomy (IgAN urine before treatment), and nine were collected after tonsillectomy (IgAN urine after treatment). Tonsillectomy has been reported to be an efficient treatment for preventing the progression of IgAN (6). According to the data in Ref. 6, tonsillectomy resulted in remission in 70.6% of IgAN patients. Nineteen patients without IgAN but with other glomerulonephritis were selected as controls (non-IgAN urine). Urine samples were obtained from 24-hr collections. These samples were stored at -80°C before analysis. Informed consent was obtained from all patients. The creatinine concentration of serum and urine, the total protein and albumin, and the volume of the urine samples are shown in Table 1.

Urine Protein Electrophoresis and Immunofixation

Sample preparation

Unconcentrated urine was used for all assays. If the urine sample contained a large amount of protein (> 500 mg/L), the sample was diluted with 0.9% (w/v)

NaCl to a total protein concentration of 200 mg/L before analysis. The semiquantitative analysis of total protein in urine was performed by our previously-described silver dot blot assay (7).

Electrophoresis and immunofixation

Using the Sample Applicator 10 (Joko, Tokyo, Japan), 0.8- μL samples were applied. The sample was electrophoresed at a constant current of 0.7 mA/cm per membrane for 25 min in veronal buffer (60 mmol/L, pH 8.6). Silver staining was carried out using our previously-described method (5).

For immunofixation, each track was overlaid with cellulose acetate strips impregnated with specific antisera against albumin and uromucoid. The immunofixed strips were washed in 0.9% (w/v) NaCl solution and stained with colloidal silver solution.

High-resolution agarose gel electrophoresis was performed according to the manufacturer's protocol.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli's method (8). A polyacrylamide gel concentration of 7% was used.

Determination of Albumin and Creatinine Concentrations in Serum and Urine, and Sodium Ion Concentration In Urine

The albumin concentration in serum and urine was determined by the immuno-turbidimetric method using N assay TIA Micro Alb (Nittobo, Tokyo, Japan). The creatinine concentration in serum and urine was determined enzymatically using the N assay CRE-L (Nittobo, Tokyo, Japan). A Hitachi 7170 automated analyzer (Hitachi, Tokyo, Japan) was used for the two measurements.

The sodium ion concentration in urine was determined using an ion-selective electrode (Olympus, Tokyo, Japan).

ELISA Procedure

Polystyrene microtiter plates (Nalge Nunc International, Roskilde, Denmark) were incubated overnight at 4°C with sheep anti-human albumin (100 L/well of

Table 1. The volume of urine and the concentration of protein, albumin and creatinine.

	Case	Concentration				Volume (L)
		Urine protein (mg/L)	Urine albumin (mg/L)	Urine creatinine (mg/L)	Serum creatinine (mg/L)	
IgAN before treatment	18	5022.2 ± 1146.9	3115.1 ± 1088.6	1555.4 ± 191.9	8.0 ± 0.2	0.5 ± 0.0
IgAN after treatment	9	6400.0 ± 2370.7	5784.4 ± 2296.4	728.9 ± 132.0	1.8 ± 1.7	1.3 ± 0.4
Non-IgAN	19	14273.7 ± 2904.7	10261.1 ± 2820.2	1170.3 ± 307.9	1.7 ± 3.9	1.0 ± 0.3

4 g/mL in coating buffer: 100 mM carbonate buffer, pH 9.4; The Binding Site). After three washings with phosphate-buffered saline (PBS)-Tween 20 (10 mM PBS containing 150 mM NaCl and 0.05% Tween 20), the plates were filled with 1% BSA (300 L/well of 1% BSA in coating buffer) to block nonspecific binding sites, and incubated for 2 hr at room temperature. After three further washings, the wells were filled with diluted (1:100) urine samples (100 L/well) and incubated for 1 hr at 37°C. After three further washings, the plates were filled with peroxidase-conjugated anti-human uromucoid IgG (0.916 g/mL in PBS, 100 L/well; The Binding Site) and incubated for 1 hr at 37°C. After three final washings, color was developed using 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Wako Pure Chemical Co., Japan) (100 L/well of 1.166 mM solution in 1 mM disodium EDTA–0.1 mM citric acid buffer pH 4.2 containing 0.011% H₂O₂) for 30 min at 37°C. Absorbance at 414 nm was measured using a microplate reader (Multi Scan Bicromatic, Dainihon Pharmaceutical Co., Saita, Japan).

RESULTS

Creatinine Concentration of Serum and Urine, Total Protein and Albumin, and Volume of Urine Samples

The creatinine concentration of serum and urine, the total protein and albumin, and the volume of the urine samples were measured. There were no significant differences among IgAN urine before treatment, IgAN urine after treatment, and non-IgAN urine.

Characteristic Urine Protein Electrophoretic Pattern: Cellulose Acetate Membrane Electrophoresis

The four selected urine protein electrophoretic patterns—IgAN urine before treatment, IgAN urine after treatment, non-IgAN urine, and the urine of a healthy subject—are shown in Fig. 1. The width of the albumin fraction was markedly expanded in IgAN urine before treatment. This phenomenon was noted only in IgAN urine before treatment, and it disappeared in IgAN urine after treatment. The width of the albumin fraction was then measured in all 46 samples using slide calipers, and the mean $\pm$ 1 SD of the width of the albumin fraction was 3.59 ± 0.03 mm (range 2.00–6.20 mm) in IgAN urine before treatment, 2.42 ± 0.40 mm (range 1.85–3.00 mm) in IgAN urine after treatment, and 2.21 ± 0.01 mm (range 1.65–3.00 mm) in non-IgAN urine. Thus the width of the albumin fraction in IgAN urine before treatment was significantly wider compared to IgAN urine after treatment ($P < 0.01$) and non-IgAN

urine ($P < 0.01$), as shown in Fig. 2. The width of the albumin fraction in 11 of 18 examples (61.1%) in IgA urine before treatment was higher than that in the IgAN after treatment and non-IgAN samples. We also sought to determine whether the width of the albumin fraction correlated with the albumin concentration in urine. As shown in Fig. 3, a reverse correlation was found when the width of the albumin fraction was compared with the albumin concentration in urine ($r = -0.352$, $P < 0.05$).

Effect of Urine Tamm-Horsfall Protein (THP) on the Width of the Albumin Fraction

The location of THP was examined using immunofixation in IgAN urine before treatment with an expanded width of the albumin fraction, and in the urine of a healthy subject. As shown in Fig. 4, the THP band was located in the same position as the albumin band in the IgAN urine before treatment having an expanded width of the albumin fraction. However, the THP band was located in the position of α_1 -globulin in the urine of the healthy subject.

Comparison of Cellulose Acetate Membrane Electrophoretic Pattern and High-Resolution Agarose Gel Electrophoretic Pattern

No expanded albumin width was detected in high-resolution agarose gel electrophoresis. This method relies on the difference in sensitivity of each silver staining.

Characteristic Urine Protein Electrophoretic Pattern: SDS-PAGE

The width of THP (90 KDa) is markedly expanded in IgAN before treatment, as shown in Fig. 5. The THP/albumin ratio (mean $\pm$ 1 SD) in IgAN urine before treatment, after treatment, and non-IgAN was 17.50 ± 2.94 mm (range 4.70–25.00 mm), 7.34 ± 0.66 mm (range 4.40–10.40 mm), and 3.72 ± 0.96 mm (range 0.00–10.50 mm), respectively. There was a significant difference between IgAN urine before treatment and IgAN urine after treatment ($P < 0.05$), and non-IgAN urine ($P < 0.01$).

Effect of Urine Sodium Ion Concentration on the Width of the Albumin Fraction

The correlation between the width of the albumin fraction and the sodium ion concentration in urine was examined. In IgAN urine before treatment, a good correlation coefficient was obtained ($r = 0.910$), but in IgAN urine after treatment and non-IgAN urine, no significant correlations were obtained ($r = 0.08$ and 0.334 , respectively). Unfortunately, sodium ion

Fig. 1. Four selected urinary protein electrophoretic patterns using (A) silver colloidal staining solution, and (B) their densitometric patterns: 1) IgAN before treatment, 2) IgAN after treatment, 3) non-IgAN, and 4) healthy subject. B: IgAN before treatment (1), and a healthy subject (4). Arrows indicate the width of the albumin fraction.

concentration could not be determined in some of the samples because they were of insufficient volume.

Measurement of THP-Albumin Complex by ELISA

The THP-albumin complex concentration in the urine samples was measured. As shown in Fig. 6, there was a

significant difference between IgAN urine before treatment and IgAN urine after treatment ($P < 0.1$), and non-IgAN urine ($P < 0.05$).

DISCUSSION

We have shown by cellulose acetate membrane electrophoresis that the width of the albumin fraction

Fig. 2. Width of the albumin fraction: 1) IgAN before treatment, 2) IgAN after treatment, and 3) non-IgAN.

Fig. 3. Correlation between the width of the albumin fraction and the albumin concentration in all 46 samples.

is significantly expanded only in IgAN urine before treatment. There is a reverse correlation between the width of the albumin fraction and the albumin concentration in urine. Thus, we speculate that albumin is combined with and/or affected by other substances in the urine. THP was detected by immunofixation in the same position as albumin, which indicates that THP combines with albumin. However, in healthy subjects THP was detected in the same position as α_1 globulin. The THP-albumin complex concentration was measured

by ELISA in IgAN urine before treatment, which revealed an overlap between IgAN before treatment, IgAN after treatment, and non-IgAN. Thus, it cannot be concluded that the THP-albumin complex is specific to IgAN before treatment. As shown above, the THP/albumin ratio in IgAN urine before treatment was significantly higher than in the other two groups. Since we suspected that this phenomenon was related to the decreased urine volume, we analyzed the urine of another renal-failure patient with decreased urine volume.

The urine samples were also examined by cellulose acetate membrane electrophoresis, and the width of the albumin fraction was measured. The mean $\pm$ 1 SD of the width of the albumin fraction was 1.47 ± 0.45 . The mean $\pm$ 1 SD of the urine volume was 0.63 ± 0.15 (L) ($n = 10$). THP was not detected by immunofixation in any of the samples. Thus, we concluded that the decreased urine volume was not the cause of THP detection in albumin fraction. In IgAN urine before treatment, there was a good correlation between the width of the albumin fraction and the sodium ion concentration. Thus, we considered that the THP-albumin complex and the sodium ion concentration affected the expanded width of the albumin fraction.

Grabska and Baginski (9) demonstrated the existence of the THP complex and some serum proteins (albumin, transferrin, and IgG) in vitro. They also suggested that the complex formation of THP is produced by desialylated and deglycosylated THP, and that such complexes have markedly elevated immunoreactivity. The carbohydrate chain of THP has gel-forming properties, and the aggregation of THP in nephron causes cast formation (10). Gel formation is increased by various factors, such as calcium ions, sodium ions, albumin, and BJPs (11–13). This phenomenon has been demonstrated in vitro, but we demonstrated it in vivo. We speculate that the expanded width of the albumin fraction (the THP-albumin complex) is affected by the carbohydrate chain of THP and is accelerated by sodium ion concentration. Since the expanded width of the albumin fraction (the THP-albumin complex) does not depend on THP (data not shown) or albumin quantity, this binding may occur due to differences in the sugar chain structure and other characteristics of THP or albumin. It is also known that sialic acid of the THP carbohydrate chain has an inhibiting action on viral hemagglutination (14–16). Suzuki and Fukuda (17) reported that *Haemophilus parainfluenzae* (HP) is more commonly isolated from the pharynx of patients with IgAN than from patients with other diseases. Furthermore, they identified the glomerular deposition of the outer membranes of HP antigens and an increased serum

Fig. 4. Detection of the THP band in IgAN urine before treatment and in the urine of a healthy subject using immunofixation and silver colloidal staining solution: 1) urinary protein electrophoretic pattern of IgAN urine before treatment, 2) albumin fraction of IgAN urine before treatment, 3) THP fraction of IgAN before treatment, and 4) THP fraction of the urine of a healthy subject.

Fig. 5. Three selected urinary protein electrophoretic patterns using SDS-PAGE: 1) IgAN before treatment, 2) IgAN after treatment, and 3) non-IgAN. Arrow indicates the THP band.

Fig. 6. Measurement of the THP-albumin complex in urine: 1) IgAN before treatment, 2) IgAN after treatment, and 3) non-IgAN.

concentration of IgA antibodies against HP antigens in IgAN. It has also been reported that IgAN is affected by other viruses and bacteria (18,19). In this study, we found that the width of the albumin fraction is significantly expanded in IgAN urine before treatment. This phenomenon was detected only by cellulose acetate membrane electrophoresis. We did not detect this phenomenon by the use of high-resolution agarose gel electrophoresis or SDS-PAGE. Further analysis is necessary to determine the mechanism that binds albumin and THP.

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