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Examination of the Molecular Diversity of α_1 Antitrypsin in Urine: Deficit of an α_1 Globulin Fraction on Cellulose Acetate Membrane Electrophoresis

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In the clinical field of nephrology, a non-invasive approach employing the analysis of electrophoretic patterns in urinary protein has been established. In this study a total of 52 urine samples with IgA nephropathy (IgAN), anti-neutrophil cytoplasmic antigen-associated crescentic glomerulonephritis (GN), and other types of GN were analyzed. Patients with high α_1 globulin (α_1G) fractions, which contained α_1AT in cellulose acetate membrane electrophoresis (CAE), tended to have α_1 antitrypsin (α_1AT) of normal molecular weight (57 kDa and 49 kDa), while patients with a deficit α_1G fraction tended to have α_1AT of low molecular weight ($<$49 kDa) ($P<0.01$). The α_1G fraction was significantly higher in patients with IgAN, and there were significantly more patients with normal molecular weight α_1AT compared to patients with other diseases ($P<0.01$). The isoelectric point of α_1AT with lower-weight molecules was more on the alkali side compared to higher-weight molecules in two-dimensional electrophoresis. Detecting changes in α_1G fractions in CAE may support the differential diagnosis of IgAN from other types of GN. J. Clin. Lab. Anal. 19:16–21, 2005.

Key words: cellulose acetate membrane electrophoresis; silver colloidal staining; urinary protein; α_1 antitrypsin; IgA nephropathy

INTRODUCTION

The purpose of urinalysis is to screen for kidney disease. Analyses of qualitative and quantitative abnormalities of urinary protein are a great help in making a rough distinction between glomerulonephritis (GN) and tubulointerstitial nephropathy. At present, a needle biopsy is indispensable for making a definitive judgment about a specific kidney disease. However, this is painful for the patient, the application of the technique depends on the patient's condition, and it is difficult to reexamine the patient in a short period of time. Therefore, an inspection method to obtain significant, accurate information from serum and urine about the pathological state of the kidney is needed. In previous studies (1,2), the Urinary Protein Expert System (UPES; Institute of Clinical Chemistry, Municipal Hospital, München-Bogenhausen, Germany) was established with the use of a dipstick test to quantify some of these characteristic single urinary proteins, and it was used as a method of judging glomerulopathy and tubulointerstitial nephropathy. However, we attempted to acquire a useful marker from urine using cellulose acetate membrane electrophoresis (CAE) to diagnosis kidney disease because of its reproducibility, technical simplicity, and rapid analytical results. To evaluate the conformity of CAE with clinical and pathological findings, we analyzed urine from patients with various kidney diseases. We previously reported some specific patterns in urinary protein fractions using CAE with a highly sensitive silver stain kit for which we improved the

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staining solution and raised the sensitivity (3–5). The increase, decrease, and disappearance of each fraction in CAE reflects certain information, such as obstacles and grades of obstacles in kidney disease. We expect this method to find clinical applications in the future for the early diagnosis of kidney disease. Therefore, it is necessary to consider in detail the relationship between clinical and pathological data for accumulated electrophoretic patterns of urinary protein in various renal diseases.

In this study, we discovered that in some types of kidney disease the α_1 globulin (α_1G) fraction disappeared or decreased. There are almost no reports regarding the disappearance and decrease of the α_1G fraction in CAE. We evaluated α_1G fractions in CAE in three groups of patients with IgA nephropathy (IgAN), anti-neutrophil cytoplasmic antigen (ANCA)-associated crescentic GN, and other types of GN to clarify the clinical implications.

The features of IgAN are hematuria and albuminuria. Pathologically, mesangium proliferation and IgA deposits in the mesangium domain can be detected by light microscopy and immunofluorescence microscopy, respectively. There is no specific inspection except for measurement of the serum IgA value. No complement is activated, although an immune complex deposits onto glomerular tissue. The grade of glomerular tissue obstacles varies widely (6–8).

The features of rapidly progressive GN (RPGN) or crescentic GN are focal segmental necrosis and epithelial cell proliferation (crescent) in most glomeruli, leading to fulminate renal failure. Associated crescentic GN (ANCA) is categorized as a Pauci-immune type, while lupus nephritis is categorized as an immune complex type. IgAN, which is diagnosed as progressive, can also be classified as the immune complex type, but is distinguished from lupus nephritis by serological inspection. There are no IgA deposits in non-IgAN, although it shows mesangium proliferation, as in IgAN. Membranous nephropathy (MN), membranoproliferative glomerulonephritis (MPGN), and minimal change disease are categorized as a nephritic syndrome characterized by heavy proteinuria. The features of MN are immune complexes seen as dense deposits on electron microscopy. The features of MPGN are duplication glomerular basement membrane (GBM) and complement activation. There is no specific pathological finding in minimal change disease (9).

MATERIALS AND METHODS

Subjects

All of the patients in this study were monitored in the Department of Nephrology and Rheumatology, Niigata University. Urine samples were obtained from 52 patients with GN who had been identified by renal biopsy specimens. Renal biopsies were performed by means of the ultrasound-guided percutaneous needle biopsy method before immunosuppressive therapy was initiated. Twenty-three patients had IgAN, nine had ANCA-associated crescentic GN, and 20 had other types of GN. Of these 20 patients, seven had non-IgAN, two had MPGN, one had MN, four had lupus nephritis, one had myloidosis, one had MDS renal disorder, one had advanced choronic GN, one had minimal change nephritic syndrome, one had thin basement membrane disease, and one had interstitial nephritis. These were classified into three groups: IgAN, ANCA, and another disease. Urine samples were obtained from 24-hr collections. These samples were stored at -80°C before analysis. Unconcentrated urine was used for all assays. Informed consent was obtained from all patients.

Quantification of Total Protein, Albumin, Creatinine, and C-Reactive Protein (CRP)

A semiquantitative analysis of total protein in urine was performed with the use of a silver dot blot assay (10). The albumin concentration in urine was determined by the immuno-turbidimetric method using an N assay TIA Micro Alb (Nittobo, Tokyo, Japan). The creatinine in serum and urine were determined enzymatically using CRE-S (Denka Seiken, Tokyo, Japan) and N assay CRE-L (Nittobo, Tokyo, Japan), respectively. The CRP concentration in serum was determined using LPIA CRP- H (Iatron, Tokyo, Japan). A Hitachi 7070 automated analyzer (Hitachi, Tokyo, Japan) was used for the measurements.

CAE

The cellulose acetate membranes used in the electrophoresis were purchased from Fuji Photo Film Co (Tokyo, Japan). With the use of the Sample Applicator 10 (Joko, Tokyo, Japan), the urine samples were applied 0.8 μL. If the urine sample contained a large amount of protein (>200 mg/L), the sample was diluted with 0.9% NaCl to a total protein concentration of 200 mg/L before analysis. The sample was electrophoresed at a constant current of 0.7 mA/cm per membrane for 25 min in a barbital buffer (60 mmol/L, pH 8.6). Silver staining was carried out with the use of our previously described method (10). The membranes were then treated with decaline, and densitometric analysis was performed with Cliniscan (Helena Institute).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Laemmli's method was adopted for SDS-PAGE (11). A total concentration of 100 mg/L was separated with the use of 12.5% polyacrylamide gels at 40 mA for 1 hr (Daiichi Pure Chemicals, Tokyo, Japan). The samples were dissolved in 0.125 mol/L Tris-HCl, 4.3% SDS, and 30% glycerol. DAIICHI III (Daiichi Pure Chemicals, Tokyo, Japan) was used as the molecular weight marker. After electrophoresis, the gels were transferred onto PVDF membranes (Immobilon-P; Millipore) in a transfer buffer (25.01 mmol/L Tris, 191.95 mmol/L glycine, and 4.0% methanol (PH 7.5)) at 180 mA for 45 min. The membranes were blocked with Block Ace (Dainihon Seiyaku, Osaka, Japan) and incubated with rabbit anti-human α_1 antitrypsin (Dako, Glostrup, Denmark) dissolved in 10 mM Tris-HCl buffer (PH 7.5) for 30 min. After three washings, the membranes were incubated with HRP-conjugated goat anti-rabbit immunoglobulin dissolved in Tris-HCl buffer for 30 min. After three more washings, bands were detected using Tris-HCl buffer containing 0.02% 3-3′ diaminobenzidine 4 HCl (Wako, Osaka, Japan) and 0.02% H_2O_2.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed with the ZOOM IPG Runner System (Invitrogen). Before electrophoresis was performed, the samples were dialyzed with a 2D Clean-Up Kit (Amersham Biosciences). The samples were dissolved in a rehydration buffer (8 mol/L urea, 2% CHAPS, 0.5% ZOOM Carrier Ampholytes, 0.002% bromophenol blue, 20 mM dithiothreitol) for one-dimensional isoelectric focusing (IEF). All protein concentrations were set at 100 mg/L. The PH range of the IEF was 3–10. After IEF was performed, the gel strips were incubated with a NuPAGE LDS sample buffer. For two-dimensional electrophoresis, Novex 4–20% Tris-Glycine ZOOM gel was used. All procedures followed the manufacturer's protocol.

Statistical Analysis

SPSS for Windows (11.0.J) software was used for statistical analysis. The Mann-Whitney U-test, Kruskal-Wallis H-test, and Yates $m \times n$ χ^2-test were used as nonparametric tests. All tests were two-tailed, and differences were statistically significant at $P < 0.05$ for the null hypothesis.

RESULTS

Comparison of Urinary Albumin, Serum Creatinine, CRP, and Creatinine Clearance (GFR) Among the Three Groups

The median and value of the 75th percentile of the albumin index in urine, which was normalized by urinary creatinine; the concentration of serum creatinine and CRP; and GFR were compared among the three groups (Table 1). There was no significant difference in albumin index among the three groups. CRP, which is an inflammation marker, was high in ANCA and another disease, while all samples were negative for IgAN.

Electrophoretic Pattern in CAE

Three typical electrophoretic patterns are shown in Fig. 1A. The α_1G fraction was detected clearly in IgAN, while in ANCA and other disease the α_1G fraction disappeared. α_1AT was identified by an anti α_1AT antibody in α_1G fraction (Fig. 1B). The α_1G fractions measured by densitometry were compared among the three groups. The α_1G fractions were normalized by an albumin fraction to avoid the influence of total protein concentration. The percentage of the α_1G fraction in IgAN was significantly higher than that in the other two groups ($P < 0.005$, $P < 0.001$) (Fig. 1C). Although 16 patients with an α_1G fraction deficit were seen among 29 patients in ANCA and other disease, in the IgAN group only two patients with α_1G fraction deficits were seen among 23 patients.

TABLE 1. Comparison of albumin, serum creatinine, CRP, and GFR among the three groups

	IgAN		ANCA		Another disease		
	Median	75%	Median	75%	Median	75%	
Urinary albumin (mg/g.cre)	635.15	967.17	736.07	1410.75	545.55	1654.28	NS
Serum creatinine (mg/dL)	0.70	0.80	1.60	2.40	0.90	1.20	IgAN vs. ANCA $P < 0.05$ IgAN vs. another disease $P < 0.05$
GFR (mL/min)	96.00	115.00	43.50	59.00	77.00	101.00	IgAN vs. ANCA $P < 0.05$
CRP (mg/dL)		–	4.10	7.10	0.00	0.25	ANCA vs. another disease $P < 0.01$

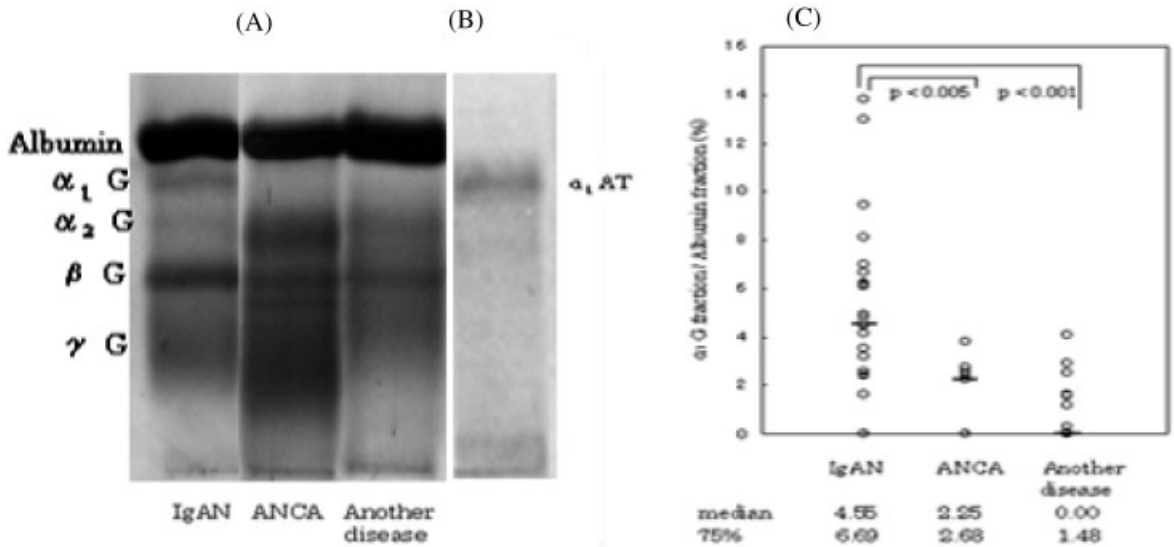

Fig. 1. **A:** CAE analysis of urinary protein (IgAN, ANCA, other disease). The α_1G fraction was detected clearly in IgAN. **B:** After CAE, each cellulose acetate membrane was transferred to a PVDF membrane, and α_1AT was detected in the α_1G position. **C:** The percentage of the α_1G/albumin fraction determined by densitometry in three diseases.

Diversity of the Molecular Weight of α_1AT

We found that the molecular weight of α_1AT in urine showed diversity in SDS-PAGE. All of the samples were classified into five types (types A–E) according to the molecular weight of α_1AT. For types A–D, α_1AT was seen at the position of 57 kDa and 49 kDa, 57 kDa, 49 kDa and other low molecules, 49 kDa only, and 49 kDa and other low molecules, respectively (Fig. 2). There were some samples in which α_1AT could not be detected by Western blotting. They were classified as type E. Other low-weight molecules contained 40 kDa, 36 kDa, and 22 kDa. The α_1AT molecule in healthy urine and serum was seen at the position of 57 kDa and 49 kDa. Also, the serum α_1AT molecule in all disease groups was 57 kDa and 49 kDa. Patients and disease groups for every molecular weight classification are shown in Table 2. There was a significantly higher number of type A in IgAN than in ANCA or other disease ($P<0.01$).

Conformity of CAE and SDS-PAGE

The relevance of the molecular weight of α_1AT and the α_1G fraction in CAE was investigated (Table 3). Three type E samples were removed from the object of investigation. In the patients with a deficit in the α_1G fractions (0%) in CAE, type A sample was 0 (a), and in the patients with high-level α_1G fractions ($\leq 5\%$), type D sample was 0 (b). That is, patients with high α_1G fractions in CAE tended to have α_1AT of high molecular weight, while patients with deficits in the α_1G fraction tended to have α_1AT of low molecular weight ($P<0.01$).

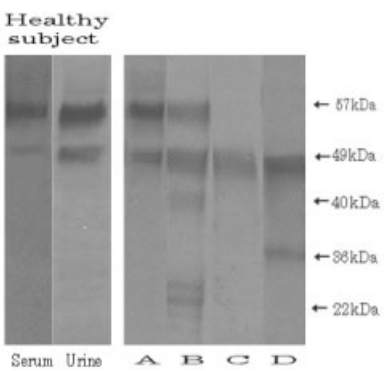

Fig. 2. SDS analysis of urinary protein shows molecule diversity (four types) of α_1AT in urine. The two sequences on the left side are α_1AT molecules in healthy urine and serum.

TABLE 2. Diversity of the molecular weight of α_1 AT

	Molecular weight of α_1 AT			Disease group		
Type	57kDa	49kDa	Other low molecules	IgAN	ANCA	Another disease
A	+	+	+	13	2	1
B	+	+		0	2	3
C		+	+	9	4	9
D		+		0	1	5
E			–	1	0	2
Total				23	9	20

TABLE 3. Conformity of CAE and SDS – PAGE

	The level of the α_1 G fraction in CAE			
Type	0%	<3%	<5%	5% ≦
A	0[a]	3	5	8
B	2	3	0	0
C	11	7	3	1
D	3	2	1	0[b]

[a]$p<0.01$, [b]$p<0.01$.

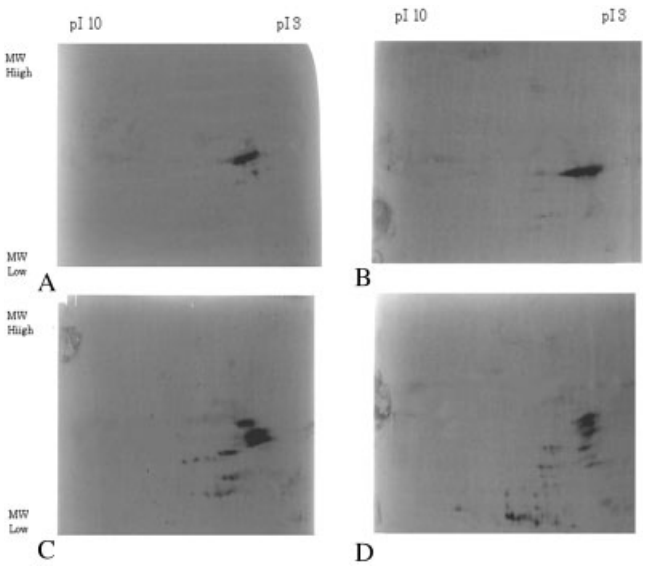

Fig. 3. Two-dimensional gel analysis of α_1AT (four types). After electrophoresis was performed, the gels were transferred onto PVDF membranes and treated with an anti-α_1AT antibody.

Two-Dimensional Gel Electrophoresis

The samples were categorized into four molecular weight classifications and analyzed by two-dimensional gel electrophoresis and immunoblotting. Representative membranes are shown in Fig. 3. In types A and B, polymerized spots were observed, and these spots were separated in the direction of low-weight molecules in types C and D. Moreover, in types C and D, some spots in the low-weight molecule domain increased, and these isoelectric points were higher than those of polymerized spots of high molecular weight.

DISCUSSION

In this study, it was clear that the α_1G fraction in ANCA and another disease was significantly lower than that of IgAN in CAE, and that this phenomenon was due to the molecular diversity of α_1AT, such as the change from the 57 kDa band to lower molecular weight forms in SDS-PAGE. Type A could be categorized as normal, since the molecular weight of α_1AT was the same as that in healthy urine. In IgAN, the percentage of normal patterns was significantly higher than that in ANCA and another disease (IgAN: 56.5%; ANCA: 22.2%; another disease: 5%). Also, the fragmentation of the α_1AT molecule occurred specifically in urine.

The lower α_1AT (<57 kDa band) in some samples was not detected in the position of the α_1G fraction CAE; however, they had a high concentration by densitometric analysis of SDS-PAGE (data not shown). The isoelectric point of α_1AT with lower-weight molecules was more alkali than that of higher-weight molecules in two-dimensional electrophoresis.

High-resolution agarose gel electrophoresis and silver staining were performed with the use of a Titan Gel Silver Stain Kit (Helena Institute). In agarose gel electrophoresis, the α_1G fraction showed the same phenomenon as CAE. However, since the minimum detection sensitivity of the silver staining solution suitable for agarose gel is 300–500 mg/L, samples with low protein concentrations must have been concentrated. On the other hand, the method we adopted can be carried out without a concentration operation, since the minimum detection sensitivity is 2.5 mg/L. This leads to a time reduction, and the influence of the concentration operation on the fraction pattern is avoided.

There was no relationship between the change from the 57 kDa band to the lower molecular weight forms and the CRP level. Ueda et al. (12) reported that oxidized α_1AT showed a change in molecular weight in serum. They demonstrated that the concentrations of oxidized α_1AT in the sera of patients with inflammatory and rheumatoid disease were higher than those in healthy subjects, and that there was no correlation between the CRP levels and oxidized α_1AT. However, oxidized AT in urine, and the relationship between the variant of molecular weight to the glomeruli obstacle have not been reported until now. α_1AT is a kind of serin protease inhibitor, and its major role is to protect tissue from proteinases such as trypsin or elastase emitted from neutrophils. In the presence of inflammation, α_1AT inactivates elastase, while myeloperoxidase, which is also emitted from neutrophils, oxidizes α_1AT. Oxidized α_1AT can not work as a protein inhibitor, so a tissue obstacle occurs (13). The cause of glomeruli in GN obstacles is the rise in GBM permeability induced by the free radicals and proteases emitted from neutrofils (14–17). Many reports have connected free radicals with GN. Lee et al. (18) demonstrated the deposition of oxidized LDL to glomeruli, and showed that it was highly observable, especially in crescentic GN

(e.g., RPGN). Moreover, they reported that although some oxidized LDL was observed in IgAN, the extent of deposition was lower than RPGN.

In the present study, the molecular weight of α_1AT showed diversity in urine, and the changes could be detected in CAE by measurement of the α_1G fraction. Generally, the inflammation and organizational damage in IgAN, except for the progressive type, are lower than those of other types of nephropathy, such as RPGN (including ANCA-associated crescentic GN and lupus nephritis). The difference in the α_1AT diversity may be due to the grade of the inflammation reaction. It is surmised that the change in the α_1G fraction in CAE indicates the grade of the nephritis obstacle. To obtain clinical data to support this hypothesis, we evaluated the relationships among the serum creatinine concentration, GFR, and α_1G fractions. The serum creatinine and α_1G fractions showed a reverse correlation ($r = -0.41$, $P < 0.01$), while the GFR and α_1G fractions showed a positive correlation ($r = 0.35$, $P < 0.05$).

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