Reduced immunoreactivity of urinary albumin in patients with cardiovascular diseases: Analysis of immunochemically nonreactive albumin

Aki Nakayama¹, Jyunichi Nishimaki¹, Tokuhiro Kawara¹, Takeshi Kasama², Toshiaki Baba³, Hiroshi Yoshida³, Mitsuaki Isobe⁴, Kiyoko Shiba⁵ and Kenji Sato¹

SUMMARY

We analyzed 55 spot urine samples from patients with cardiovascular diseases. Urinary albumin concentrations were measured with size exclusion high performance liquid chromatography (HPLC), turbidimetric immunoassay (TIA) using anti human serum albumin polyclonal antibody, and enzyme-linked immunosorbent assay (ELISA) using anti human serum albumin monoclonal antibody. Fractionated urine samples from the HPLC were also analyzed with the immunoprecipitation reactions using as same monoclonal antibody as ELISA. As a result, the urinary albumin concentration analyzed by the HPLC was systematically higher than that of immunoassays, however, ‘albumin peak’ from the HPLC contained other urinary proteins. Result of immunoprecipitation reaction showed the presence of monomer albumin that could not react with the monoclonal antibody. These results suggest that not only contamination of other proteins, the albumin fraction from the HPLC included albumin with reduced its reactivity to the specific monoclonal antibody.

Key words: urinary albumin, cardiovascular diseases, immunochemically nonreactive albumin, HPLC, TIA.

INTRODUCTION

Microalbuminuria, defined as 30–300 mg of albumin per g of creatinine (30–300 mg/g Cre) in urine, is widely acknowledged as an early marker of diabetic nephropathy¹. High urinary albumin concentration was also known to be associated with endothelial dysfunction in patients with or without diabetes mellitus²–⁵. In non-diabetic patients with hypertension, it was shown that the subjects with microalbuminurea had significantly higher prevalence of coronary artery related or other cardiovascular diseases than the normalalbuminuric subjects⁶.

Urinary albumin gets fragmented into peptides during the renal passage, and is subsequently filtered by the glomerules. Consequently, the normal urine contains both the albumin-derived peptide fragments and a small amount of the intact albumin (66 kDa)⁷–¹⁵. Currently used immunological assays or dye-binding assays might not detect these albumin fragments⁹–¹¹.

In 2001, Greive et al. reported that conventional immu-
no assays underestimated the urinary albumin concentrations in diabetic rats because the commercially available anti human albumin antibodies were unable to detect intact molecular weight but immunochemically nonreactive albumin\cite{19}. Immunochemically nonreactive albumin was also found in the urine of diabetic patients by size exclusion chromatography (HPLC) analysis, even though the total intact albumin, which contains both immuno-reactive and immuno-nonreactive monomer albumin, could be measured by this method\cite{17-18}. Furthermore, Comper et al. reported that in patients with type 1 and type 2 diabetes, the total intact albumin measured using the HPLC method could predict the onset of persistent albuminuria approximately 2–4 years earlier than the conventional radioimmunoassay\cite{19}.

Although recent studies have established a relationship between the urinary albumin excretion and cardiovascular risk, the reason for the variability in albumin reactivity to the anti albumin antibody is not understood.

In the present study, we analyzed the urinary albumin concentration using the HPLC method, TIA and ELISA to characterize the immunochemically nonreactive albumin. In addition, we wanted to clarify why the urinary albumin in patients with cardiovascular diseases shows variable reactivity to commercially available anti albumin antibody.

**MATERIALS AND METHODS**

**Subjects**

Analysis was performed on spot urine samples from 55 patients (31–83 years old) with cardiovascular diseases (hypertension, hyperlipidemia, arrhythmia, ischemic heart disease and myocardial infarction) who consulted the Tokyo Medical and Dental University Hospital Faculty of Medicine. Patients with complications of diabetic mellitus were excluded from this study. We also analyzed spot urine samples from 55 healthy volunteers (20–57 years-old). Verbal informed consents were obtained from all subjects. All urine samples were stored at –80°C before analyses.

**HPLC**

The urinary albumin concentration was determined with a Waters HPLC System (pump: 600E, auto sampler: 486, software: Millenium 32, Waters Corporation, Milford, MA, USA) using the Accumin\textsuperscript{TM} (TOTAL INTACT ALBUMIN ASSAY\textsuperscript{TM}, AusAm Biotechnologies, Inc., New York, NY, USA) and following the procedure described by Comper et al.\cite{20}. The lower limit for the detection of albumin using this method was 3 mg/L.

**TIA**

The urinary albumin was measured with a HITACHI7070 Automatic Analyzer (Hitachi, Tokyo Japan) using a commercially available immunoturbidimetry reagent kit (N-assay TIA micro Alb, Nitto Boseki Co., Tokyo, Japan), and following the manufacturer’s instruc-

**ELISA**

Nunc MaxiSorp 96-well flat-bottomed immunoplate was coated with the mouse anti human albumin monoclonal antibody (Zymed Laboratories Inc, South San Francisco, CA, USA) at 0.5 μg/ml in 0.1 mmol/L sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. This monoclonal antibody was also used following Western blot and immunoprecipitation assay. The wells were washed with 0.05% Tween containing PBS, blocked with 0.5% casein containing PBS, and subsequently incubated for 1.5 h at room temperature with urine samples (diluted 1:100 or 1:1000 in PBS). After washing, 1:10000 diluted rabbit anti human albumin polyclonal antibody (DAKO Cytomation, Carpinteria, CA, USA) was added to each well as the detection antibody and incubated for 1.5 h at room temperature. Wells were washed with PBS and then incubated with 1:10000 diluted peroxidase-conjugated goat anti rabbit IgG antibody (DAKO Cytomation, Carpinteria, CA, USA) for 1.5 h at room temperature. Finally, after washing three times with PBS, the wells were incubated with the peroxidase substrate (Sure Blue Reserve\textsuperscript{TM} TMB Microwell Peroxidase Substrate, KPL, Gaithersburg, MD, USA) for 15 min. The reaction was stopped by the addition of 16.7 mmol/L sulfuric acid, and the absorbance was measured at 450 nm. The calibration curve for the human serum albumin standard (Sigma-Aldrich Co., St.Louis, MO, USA) was linear in the range of 0.156–1.25 mg/L with a correlation coefficient >0.998.

**Total urinary protein and creatinine assays**

The semiquantitative analysis of total urinary protein was determined using a silver dot blot assay\cite{21}. The urinary creatinine concentration was measured with HITACHI7070 Automatic Analyzer using the commercially available enzyme reagent kit (N-assay CRE-E, Nitto Boseki Co., Tokyo, Japan). For each urine sample, the ACR was calculated using the individual albumin and creatinine concentrations. A goat anti human albumin polyclonal antibody was included in the immunoassay kit. The lower limit for the detection of albumin using this method was 5 mg/L.

**Electrophoresis**

SDS PAGE was carried out following the method of Laemmli\cite{22} and using 12.5% uniform polyacrylamide gels (PAG Mini “DAIICHI” 12.5, Daichii pure chemicals, Tokyo Japan). Each sample was diluted with PBS, and approximately 0.6 μg protein was mixed with equal volume of the sample buffer (for reducing SDS PAGE: SDS βME sample buffer; for nonreducing SDS PAGE: Tris SDS sample buffer; Daichii pure chemicals, Tokyo, Japan). The sample for the reducing SDS PAGE was denatured for 5 minutes at 100°C. The running buffer used was SDS-Tris-glycine...
running buffer (Daiichi pure chemicals, Tokyo Japan).

After electrophoresis, the gel was stained with a silver staining kit (Silver stain 2 kit WAKO, Wako Pure Chemical Industries, Ltd, Osaka, Japan). The albumin percentage for each band was calculated by PC densitometric analysis (Scion Image Beta 4.02 Win, Scion Corporation, Maryland, USA).

**Western blot analysis**

The SDS PAGE separated proteins were transferred from the gels to the PVDF membrane (Immobilon-P, Millipore, Bladford, MA, USA). Each membrane was first incubated in the blocking solution (Block Ace, Dainippon Sumitomo pharma Co., Osaka, Japan) for 1 hr. After blocking, the membrane was washed three times with the washing buffer (10 mmol/L Tris, 0.9% NaCl, 0.05% Tween 20, pH 7.5), and then the membrane was incubated with the anti human albumin monoclonal antibody (Zymed Laboratories Inc, South San Francisco, CA, USA) or rabbit anti human transferrin antibody (DAKO Cytomation, Carpinteria, CA, USA) for 1 hr. After that, the membrane was incubated with the peroxidase-conjugated rabbit anti mouse IgG antibody (DAKO Cytomation), or peroxidase-conjugated goat anti rabbit IgG antibody (DAKO Cytomation) for 1 hr. Subsequently, the immunoreactive band on the membrane was visualized following treatment with the DAB solution (0.02% 3-3’ Diaminobenzidine 4HCl 0.02% H2O2).

**MALDI-TOF mass spectrometry analysis of the HPLC fractionated sample**

MALDI-TOF mass spectra were acquired using the Voyager DE-STR spectrometer (Applied Biosystems, Framingham, MA, USA).

The albumin peak was fractionated by the HPLC and desalted using a Nap-5 column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA). The desalted sample was mixed with equal volume of sinapinic acid in a mixture containing 30% acetonitrile: 70% water: 0.1% TFA, the mixture was spotted on the sample plate and air dried. Then, the mixture sample was analyzed by MALDI-TOF mass spectrometry in positive linear mode.

**Immunoprecipitation reaction using the anti human albumin monoclonal antibody**

The HPLC fractionated albumin sample was also subjected to immunoprecipitation reaction. In brief, 100 μl of the fractionated albumin sample (diluted with PBS under 5 μg/ml), 5 μl of the mouse anti human albumin monoclonal antibody, the same as that used for above ELISA first reaction, and 20 μl of Protein A sparse TM Fast Flow beads (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA) were mixed all together and incubated overnight at 4°C. The reaction mixture was then centrifuged (8,000 rpm, 1 min) to separate the immunoprecipitate and supernatant as a non-reactive fraction. To eliminate any free albumin or antibody, the immunoprecipitate was washed three times and the resulting 2nd supernatant was obtained. The immunoprecipitate and supernatant fractions were analyzed with reducing SDS PAGE and Western blot following above methods. In Western blot, the mouse anti human albumin monoclonal antibody used in the immunoprecipitation reaction was employed as a primary antibody to investigate antigen (albumin) excess and the secondary antibody was the peroxidase-conjugated rabbit anti mouse IgG antibody. In this method, 5 μg/mL of the commercially available human serum albumin (Sigma-Aldrich Co., St.Louis, MO, USA) bound completely to the antibody, and was not found in the supernatant (data was not shown).

**Identification of the domain recognized by the anti human albumin monoclonal antibody using Western blot analysis**

To identify the domain recognized by the mouse anti human albumin monoclonal antibody used for the ELISA first reaction, and the immunoprecipitation assays, we digested the human serum albumin with cyanogens bromide (CNBr) and then analyzed the digested fragments by Western blot.

Briefly, 200 μl of 20 mg/ml human serum albumin (Sigma-Aldrich Co., St.Louis, MO, USA) solution was diluted with 70% formic acid and then incubated with 2 μl of 2-mercaptoethanol (Sigma-Aldrich Co., St.Louis, MO, USA) for 48 hrs at 37°C. Next, 200 μl of 200 mg/ml CNBr was added to this solution and further incubated for 72 hrs at 37°C.

After CNBr digestion, the solvent was changed to PBS, and the digested sample was analyzed by reducing SDS PAGE. After electrophoresis, the gel was stained with CBB (Quick CBB, Wako Pure Chemical Industries, Ltd, Osaka, Japan). The protein fragments were then subjected to Western blot analysis using the anti human albumin monoclonal antibody.

**Statistical analysis**

SPSS for Windows (11.0J) software was used for the statistical analysis. Spearman’s rank correlation test was used for investigating the correlation. The passing and Bablok method was used for comparing different methods. A p value of <0.01 (p<0.01) was considered to be statistically significant. All measured albumin concentrations are expressed as the mean±SE.

**RESULTS AND DISCUSSION**

The aim of this study was to investigate the altered immunoreactivity of urinary albumin in patients with cardiovascular diseases. Our findings suggest that monomer albumin, which could not be detected by a monoclonal antibody, is excreted in the urine of cardiovascular patients.

The HPLC results tended to measure the albumin con-
concentration as being systematically higher than the immunochromatographic methods. In the patient group, the urinary albumin concentration ranged between 3.00–1685 mg/L (mean±SE=83.7±32.2 mg/L) as determined by the HPLC method, between 1.00–1110 mg/L (mean±SE=57.7±21.8 mg/L) as determined by TIA, and between 0.52–795 mg/L (mean±SE=32.4±14.5 mg/L) as determined by ELISA. On the other hand, in the healthy volunteer group the urinary albumin concentration ranged between 4.00–30.0 mg/L (mean±SE=14.0±0.976 mg/L) as determined by the HPLC method, between 1.10–16.9 mg/L (mean±SE=6.23±0.553 mg/L) as determined by TIA, and between 1.60–55.5 mg/L (mean±SE=14.8±1.71 mg/L) as determined by ELISA.

Based on the results of TIA and ELISA analysis, the ACR was classified as normoalbuminuria (<30 mg/g Cre), microalbuminuria (30–300 mg/g Cre), and macroalbuminuria (>300 mg/g Cre). In this study, we have followed the nonparametric reference interval classification of the ACR results from HPLC as established by Owen et al. (22–250 mg/g Cre for girls, 20–130 mg/g Cre for boys, 14–62 mg/g Cre for women, and 10–37 mg/g Cre for men). Following these, in this study the ACR results from HPLC were classified as normoalbuminuria (results falling within the reference interval of each gender and age) and albuminuria (results higher than normoalbuminuria).

Table 1 compares the classifications of subjects according to the ACR results obtained from HPLC, TIA and ELISA. As shown, the classifications based on the ACR results obtained using different methods were in fairly good agreement with each other. However, there were also some disagreements—for example, in a few cases, the subjects classified as normoalbuminuria by TIA and ELISA were classified as albuminuria by HPLC.

There were also disagreements between the ACR classification obtained from TIA and ELISA in the case of 6 patients (10.9%) and 7 healthy volunteers (12.7%). In these cases, the ACR values obtained from the ELISA were lower than those from TIA in the patients group, but were higher than those from TIA in the healthy volunteer group. Furthermore, these ACR values by ELISA were close to those obtained from HPLC in the healthy group.

ACR results based on the HPLC analysis revealed that the urine samples from two patients with cardiovascular diseases were 1.5 times or higher than those from TIA and ELISA (ACR value of sample number 1: HPLC 1051 mg/g Cre, TIA 692 mg/g Cre and ELISA 496 mg/g Cre; ACR value of sample number 2: HPLC 72.9 mg/g Cre, TIA 8.89 mg/g Cre, and ELISA 2.90 mg/g Cre). Thus, we decided to focus our attention on determining the underlying cause for this deviation.

First, we fractionated the monomer albumin peak from these two samples by HPLC. Figure 1 shows the HPLC profiles of samples no. 1 and no. 2. The monomer albumin fraction from each sample was then analyzed by nonreducing and reducing SDS PAGE (Fig. 2). Under both conditions, the major protein band was the monomer albumin in both samples (Fig. 2a and b). However, two other higher molecular weight protein bands were also visualized in sample no. 1 (Fig. 2a). These two bands were identified as the albumin dimer and transferrin by Western blot analysis (data not shown), and their identities were further confirmed by MALDI-TOF mass analysis (Fig. 3).

Under reducing conditions, we observed a decrease in the intensity of the monomer albumin band and a concomitant appearance of lower molecular weight protein bands on the SDS PAGE gel (Fig. 2b). These results indicate that one of the reasons for the overestimation or disagreement of ACR values is the presence of other contaminating proteins in the HPLC method. In another study, Sviridov et al. suggested that α1-acid glycoprotein or α1-protease inhibitor could be contaminants in the HPLC analysis. In sample no. 1, the urinary albumin concentration was sufficiently high that it was classified as macroalbuminuria (>300 mg/g Cre); thus, contamination of the albumin fraction with other proteins isolated from the HPLC might be significant.

To further characterize the HPLC fractionated monomer...
albumin peak from sample no. 1, the HPLC fractionated albumin was analyzed by an immunoprecipitation assay, and the immunoprecipitate and the two supernatant fractions were analyzed by SDS PAGE. The results of the SDS PAGE analysis are shown in Fig. 4. The albumin bands are shown within the dotted box. The IgG derived bands were clearly detected by silver stain. The IgG in the immunoprecipitate (lane 1) that was initially bound to the protein A Sepharose, which showed up as the heavy chain and light chain in the SDS PAGE analysis. The IgG band found in the supernatant fractions (lanes 2 and 3) represents the unbound IgG. The IgG derived bands were visualized by the reaction with peroxidase-conjugated rabbit anti mouse IgG antibody in the Western blot analysis (lanes 4–6). In this method, the commercially available human serum albumin (Sigma-Aldrich Co., St.Louis, MO, USA) bound completely to the antibody, and was not found in the supernatant fraction (data not shown).

As shown in Fig. 4, the albumin band present in the immunoprecipitate was stained with silver stain in the SDS PAGE and was also detected by the anti human albumin monoclonal antibody in the Western blot assay. In contrast, although the albumin present in the supernatant fractions was clearly stained with the silver stain in SDS PAGE (lanes 2, 3), it was not recognized by the monoclonal antibody in Western blot assay (lanes 5, 6). Clearly, the 66 kDa protein in the supernatant fractions could not have come from the excess antigen, but the non-reactive antigen, would not have been detected by the antibody in the immunoprecipitation reaction. Furthermore, the protein was not detected by anti human transferrin polyclonal antibody, anti human α₁-antitrypsin polyclonal antibody, or anti human α₁-microglobulin polyclonal antibody in the Western blot assays (data not shown). Therefore, the 66 kDa protein remaining in the supernatant fractions was likely to be monomer albumin.
Figure 5 shows the results of the SDS PAGE and Western blot analyses of CNBr digested albumin. The SDS PAGE was run under reducing condition and the anti human albumin monoclonal antibody was used for the Western blot analysis. Theoretically, CNBr should digest albumin into seven peptides. As shown in Fig. 5a (lane 3), several of these peptide fragments were visible in the stained SDS PAGE gel. The monoclonal antibody recognized a ~20 kDa peptide consisting of the amino acid residues 124–298 of human albumin (Fig. 5c). This monoclonal antibody was used in the ELISA, and immunoprecipitation reactions. Thus, this result showed that urinary albumin lost the structural domain containing at least the antibody epitope and may be contained in urine sample no. 1. Furthermore, these alterations of albumin may be related to disagreements of ACR values between TIA and ELISA.

We also observed a decrease in the amount of monomer albumin in the reducing SDS PAGE. This result suggests that at least part of the urinary albumin in cardiovascular disease becomes fragmented under reducing condition. Our results are partly congruent with those reported by Osicka et al. and we have previously observed a similar decrease in the albumin content in patients with diabetic mellitus. The degree to which this unstable albumin relates to the decreased reactivity of the antibody is at present unclear and further examination is warranted. Additional investigations using other characterized anti albumin antibodies are also needed to clarify why a portion of urinary albumin is immunochemically non-reactive.

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