The Korean Journal of Internal Medicine: 19:141-148, 2004

Conformational Transitions and Glycation of Serum Albumin in Patients with Minimal-Change Glomerulopathy

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Background : There has been a lack of study on the structural changes of serum albumin in patients with minimal change disease (MCD). To determine whether glycation and/or conformational transitions of albumin are involved in the pathogenesis of albuminuria, nine patients with MCD were enrolled in a prospective follow-up study for comparison of these parameters in serum albumin during the remission and relapse of nephrotic syndrome.

Methods : Circular dichroism measurements were made with purified albumin. Ellipticities at each wavelength were transformed to mean residue ellipticity. Monosaccharide composition was analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection.

Results : There was no difference in the proportions of α-helix, β-conformation, and β-turn of albumin between the sera of control patients and those with nephrotic syndrome. However, the proportion of the random configuration was slightly higher in the plasma albumin of patients in relapse than in those in remission. The proportion of the random configuration was lower in the albumin of the serum than in the urine of patients with nephrotic syndrome, but there was no difference in the proportions of α-helix, β-conformation, and β-turn of albumin between their plasma and urine.

Conclusion : Our results suggest that conformational changes in albumin are involved in albuminuria in patients with MCD.

Key Words : Glycosylation, Protein Conformation, Serum Albumin

INTRODUCTION

Minimal-change glomerulopathy (or minimal-change disease, MCD) is a histopathologic lesion that is almost always associated with nephrotic syndrome at the disease onset[1]. It accounts for about 80% and 20% of cases of nephrotic syndrome in children and adults, respectively[2]. MCD appears under light microscopy as a lack of definitive alteration in glomerular structure, but the most obvious and consistent finding is a characteristic fusion of the epithelial foot processes observed by electron microscopy[1, 2]. Urine principally contains albumin and minimal amounts of high molecular weight proteins such as IgG and β_2-macroglobulin. This selective proteinuria in conjunction with foot-process effacement has lead to a consensus that the main lesion of MCD is damage to podocytes and loss of the fixed negative charge in the glomerular filtration barrier for proteins[3]. However, the precise pathogenesis of MCD is still unclear. MCD is highly steroid responsive, and approximately 90% of childhood cases and 50% of adults cases enter remission following a high-dose oral glucocorticoids

• Received : January 26, 2004
• Accepted : April 14, 2004
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treatment[1, 2]. After the clinical response to initial treatment, as few as 25% of patients exhibit long-term remission, 25% to 30% have infrequent relapses, and the remainder have frequent relapses, steroid dependence, or steroid resistance[3]. The molecular mechanisms underlying this unresponsiveness, however, are unknown. The heterogeneity of the responses to glucocorticoid raises questions about the precise mechanism of the steroid on the remission of nephrotic syndrome. Bantel et al.[4] reported that steroid resistance is associated with increased epithelial activation of NF-Kappa B. However, the precise mechanisms of the steroid response and/or unresponsiveness are unknown. One way to elucidate the pathogenesis of MCD may be to understand the role of glucocorticoid in the changes not only in the glomerular filtration barrier, but also in the conformational transitions of serum albumin induced during the clinical course of nephrotic syndrome. Studies have shown that glucocorticoids diffuse passively through the cell membrane and bind to specific sites on glucocorticoid-regulated genes, thus altering levels of transcription[5, 6]. It has been postulated that the anti-inflammatory effects of glucocorticoids result from glucocorticoid-receptor-mediated inhibition of the transcription factors that normally stimulate the activity of various cytokine genes[7, 8]. In contrast, Regele et al.[9] reported an opposite effect of glucocorticoids - an upregulation of the expression of the dystroglycan complex in cultured muscle cells. These findings suggest that the effect of glucocorticoid on the regulation of a gene expression is more complicated than currently thought. The Structural Classification of Proteins database provides a detailed and comprehensive description of the relationships of all known protein structures[10]. In general, the simplest arrangement is a helical structure, in which the polypeptide backbone is tightly wound around an imaginary axis drawn longitudinally through the middle of the helix[11, 12]. The so-called α-helices, are distinguished as being common and relatively invariant secondary structural elements of proteins. These are not rigid bodies;, their deformations can have significant effects on protein function[13].

A more extended conformation is the β-conformation in which the backbone of the polypeptide chain is extended into a zigzag that can be arranged side by side to form a structure resembling a series of pleats[14]. In globular proteins, which have a compact folded structure, nearly one-third of the amino acid residues are in turns or loops at which point the polypeptide chain reverses direction. These are the connecting elements that link successive runs of α-helix or β-conformation. Particularly common, are β-turns that connect the ends of two adjacent segments of an antiparallel β-sheet[15, 16]. Recent evidence suggests that a protein can fold into an alternative three-dimensional structure, but the reasons for this have not yet been identified. Such "misfolding" leads to a loss of the normal function of a protein[17, 18].

Another aspect of the albumin molecule to be addressed is glycation after a gene expression. In general, the addition and subsequent processing of oligosaccharides is the principal chemical modification to most plasma membranes and secretory proteins during their synthesis in the Golgi cisterna. As a consequence, most proteins contain one or more oligosaccharides. However, serum albumin is a nonglycoprotein since it has no site for glycation on its amino acid sequence. Shaklai et al.[19] reported that *in vivo* glycated albumin exhibits a 50% reduction in its affinity for bilirubin and of 95% affinity reduction for the highly unsaturated cis-parinaric acid. Furthermore, they provided evidence of a albumin conformational change that reduced the quantum yield of tryptophan fluorescence by 30%. Glycation increases the negativity of albumin and it promotes more rapid uptake into isolated endothelial microvessels[20], into the endoneurium of rat sciatic nerve[21], and across renal glomerular basement membranes in an *in vitro* model[22]. Doublier et al.[23] suggested that glycated albumin contributes to nephrin downregulation, which modulates glomerular barrier selectivity. It has also been shown to stimulate TGF-beta 1 production and protein kinase C activity in glomerular endothelial cells[24]. Cohen et al.[25] proposed that normalization of the plasma glycated-albumin concentration amelioratesd glomerular structural and functional abnormalities. However, most of these studies have been confined to diabetic animals or diabetic patients. These results led us to hypothesize that unusual glycation and/or conformational transitions of albumin is involved in the pathogenesis of albuminuria and is influenced by glucocorticoid treatment in patients with MCD. This study was designed to observe changes in the conformations and glycation of albumin in patients with MCD during the remission and/or relapse of nephrotic syndrome.

METHODS AND MATERIALS

1. Study Design

Nine patients with nephrotic syndrome associated with MCD were enrolled in a prospective follow-up study for the comparison of the conformations and glycation of serum albumin during the remission and relapse of nephrotic syndrome for 1 year. All patients were initially treated for nephrotic syndrome using the routine regimen described below. Patients were scheduled to visit the outpatient clinic every month, and protein levels in urine were measured qualitatively (dipstick) or quantitatively (24-h urine). The Ethics Committee of Soonchunhyang University Chonan Hospital approved the study design. Informed written consent was obtained from all subjects, and their participation was voluntary.

Table 1. Physical and biochemical characteristics of patients with MCD and of the control at the onset of the study.

Characteristics	MCD	Control
Age (years)	33.1+9.4	32.0±10.3
Body weight (kg)	63.5±3.9	60.9±4.7
Height (cm)	168.6±4.25	167.5±8.1
Systolic BP (mmHg)	126.1±6.5	117.5±9.5
Diastolic BP (mmHg)	81.4±3.1	80.5±2.5
Hemoglobin (mg/dL)	12.4±1.1	13.2±0.8
Fasting blood glucose (mg/dL)	96±12.9	90.3±4.5
Serum albumin (g/dL)*	2.7±0.7	3.8±0.3
Serum creatinine (mg/dL)	0.9±0.1	1.0±0.2
Cholesterol, total (mg/dL)**	364.3±68.9	194.5±33.1
Triglyceride (mg/dL)**	294.8±150.6	143.8±27.5
Urine protein (mg/24 h)**	6977.1±4230.6	102.5±12.6
Urine creatinine (mg/24 h)**	854.4±208.7	808.8±51.2

*p<0.05, **p<0.01

Plasma samples were obtained from an antecubital vein early in the morning after fasting overnight. The serum of relapse patients was drawn when the level of urine albumin was at a peak, and the serum of remission patients was obtained when the level of albumin decreased below 250 mg/24h. The serum and urine samples used for studying the conformations and glycation of albumin were stored at -20℃ and assayed within 1week. We found that freezing and thawing to and from -20℃ did not influence the results of conformation investigations.

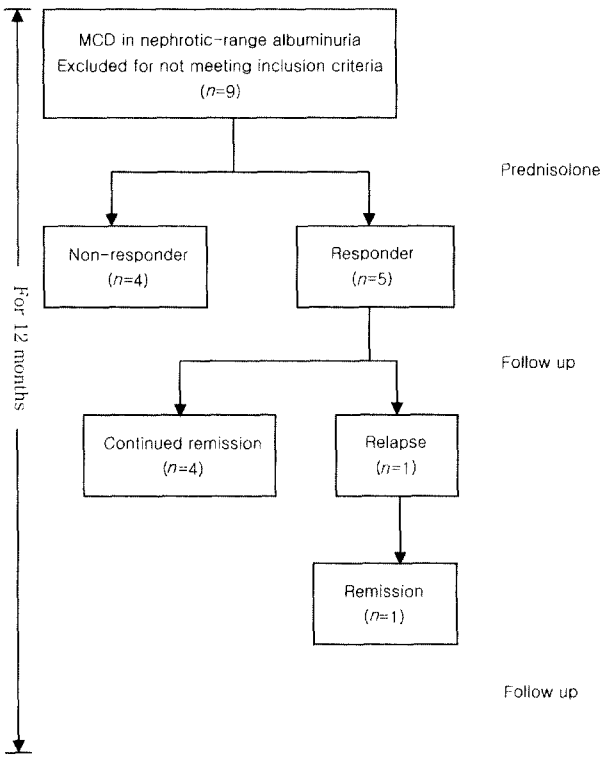

Figure 1. Flow diagram of the longitudinal follow-up time for sampling.

2. Study subjects

Seven healthy subjects (control group; four males, three females; age 43.2±11.5 years) and nine patients with MCD (eight males, one female; age 33.1±9.4 years, mean±SD, Figure 1) were enrolled for the blood albumin study. The control group was comprised of healthy volunteers recruited during a regular physical examination at Soonchunhyang University Chonan Hospital Health Promotion Center (Chonan, Korea).

MCD was confirmed by a renal biopsy. In all cases the glomerular size and architecture were normal under light microscopy, and immunofluorescence investigations were typically negative for immunoglobulin and C3. Electron microscopy revealed the characteristic diffuse effacement of the foot processes of visceral epithelial cells.

Exclusion criteria were hypertension, diabetes, and any kind of malignancy. None of the patients had a history of treatment with nonsteroidal anti-inflammatory drugs, and none had received drugs other than glucocorticoid or cyclophosphamide within 1 month of the blood and urine sampling.

The clinical and physical characteristics at the onset of the study of the nine patients with MCD are summarized in Table 1. Our routine treatment regimen for MCD cases complicated with nephrotic syndrome was 8 weeks of oral glucocorticoid: 1 mg/kg body weight per day for 4weeks, followed by 1 mg/kg body weight per day on alternate days for 4weeks. When there was no remission of urine protein to below 50% of the initial level observed, we considered this as steroid resistance and hence steroid replacement was discontinued. If the amount of protein decreased to less than 250 mg/24h, this was considered a remission. If the urine albumin was between 250 mg/24h and 50% of the initial level, we considered this a partial remission which was then treated for a further 2 months with a combination of glucocorticoid (1 mg/kg) and cyclophosphamide (2 mg/kg).

Case 1 was a 45-year-old male who had been diagnosed with MCD by renal biopsy 6 months before the initiation of the study. His clinical course was as an infrequent relapser. At the beginning of this study, he was in a nephrotic syndrome state nephrotic syndrome. The second remission occurred 3 months later, which was treated with glucocorticoid throughout the observation period.

Case 2 was a 23-year-old male. He was admitted for a renal biopsy 24 months before the initiation of this study, because of a massive urine protein level of 12.6 g/24h. He had relapsed with nephrotic syndrome twice during the previous 24months. At the initiation of this study, he was on the third relapse of nephrotic syndrome. A remission was followed by a

3-month regimen of prednisolone with cyclophosphamide that continued throughout the study.

Case 3 was a 33-year-old male. He was diagnosed as a patient with MCD showing nephrotic syndrome 1month before the initiation of the study. He was receiving a glucocorticoid treatment; when a remission occurred 4months after commencing the treatment of glucocorticoid, which was then sustained to the end of the observation period.

Case 4 was a 32-year-old male. He had been diagnosed with MCD 10 years before this study. During the intervening period he had been an infrequent relapser, with three relapses of nephrotic syndrome. At the beginning of this study he was showing nephrotic syndrome with a urine albumin level at 6.9 g/24h. A remission followed again 3months after a treatment ofwith prednisolone, and this was continued till the end of the observation period.

Case 5 was a 23-year-old male. He was diagnosed as a patient with MCD showing nephrotic syndrome 1month before the initiation of the study. A relapse of albuminuria was followed 4months after a routine treatment. A blood sample was taken again at this point. In November, 2002, nephrotic-range proteinuria recurred again and the second serum and urine samples were obtained. During the observation period, he was an infrequent relapser retrospectively.

Cases 69 were steroid nonresponders. They were diagnosed with MCD several months before the initiation of this study (mean, 6months; range, 2~10months). Their urine albumin levels were 2.0~4.5 g/24h during the period of study. Blood and urine samples were obtained when the urine albumin level was over 3.5 g/24h.

3. Laboratory assays

Complete blood count, blood chemistry including fasting blood glucose, serum urea nitrogen, serum creatinine, serum albumin, total cholesterol, and triglyceride were measured during every visit forin all subjects. Twenty-four-hour urine was collected for 24h from 8 a.m. into a bag containing thymol. Blood samples were drawn in close proximity to the 24-h urine collection period after fasting overnight.

4. Albumin isolation by chromatography

One milliliter of human serum was buffer exchanged on a PD-10 column (Amersham Biosciences, Uppsala, Sweden) to 50 mM potassium phosphate, pH 7.0, which was then used for the equilibration of Blue Sepharose (Amersham Biosciences). The serum was passed through 5 mL of Blue Sepharose in a small empty column that was subsequently washed with five column volumes of the equilibration buffer. Albumin was eluted with two column volumes of the same buffer containing 1.5 M potassium chloride. For further purification, albumin fractions were subjected to gel-filtration chromatography (Figure 2) with Superdex 200 pg (16×60 cm, Amersham Biosciences). The solvent was 50 mM sodium phosphate, pH 7.5, and it contained 0.15 M sodium chloride. The flow rate was 1.0 mL/min, and the eluting proteins were monitored at 280 nm. The final fraction of albumin was confirmed by SDS-PAGE (Figure 3).

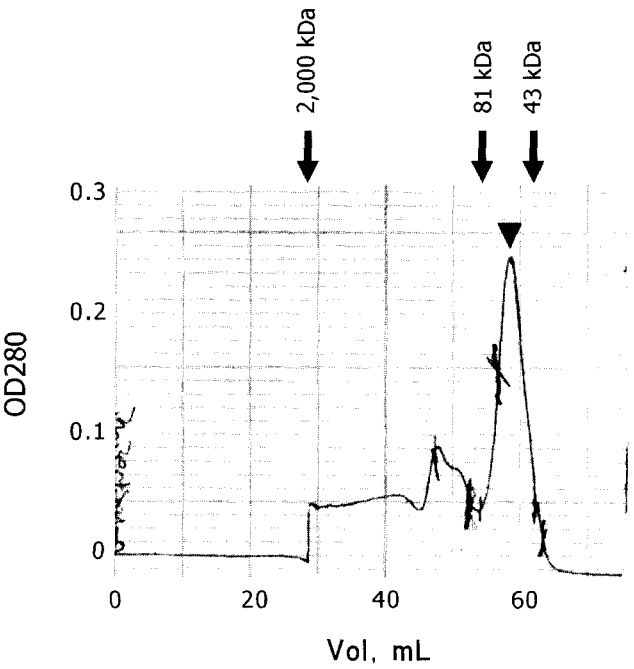

Figure 2. Isolation of human serum albumin by gel-filtration chromatography. Albumin fraction in Blue-Sepharose chromatography was used for gel-filtration chromatography under the conditions described in the Methods and Materials section. The positions of molecular-size markers and albumin are indicated by arrows and the arrowhead, respectively.

5. Circular dichroism measurements and analysis

Circular dichroism spectra were determined at room temperature using a spectropolarimeter (Jasco J-715, Tokyo, Japan). Fractionated albumins were extensively dialyzed against 10 mM sodium phosphate buffer, pH 8.0. Circular dichroism measurements were made with the purified albumin in 10 mM sodium phosphate buffer, pH 8.0, at a protein concentration of 350 g/350 l. The spectral bandwidth was set at 1 nm. The cuvette used gave a light path length of 1 mm over 200~250 nm. The scan speed was set to 50 nm/min (Figure 4). All scans were carried out five times and averaged to mean values. The solvent spectrum was manually subtracted from the protein spectrum. Ellipticities of each wavelength were transformed to mean residue ellipticity. The content of the secondary structure was determined using the method described by Yang et al[26].

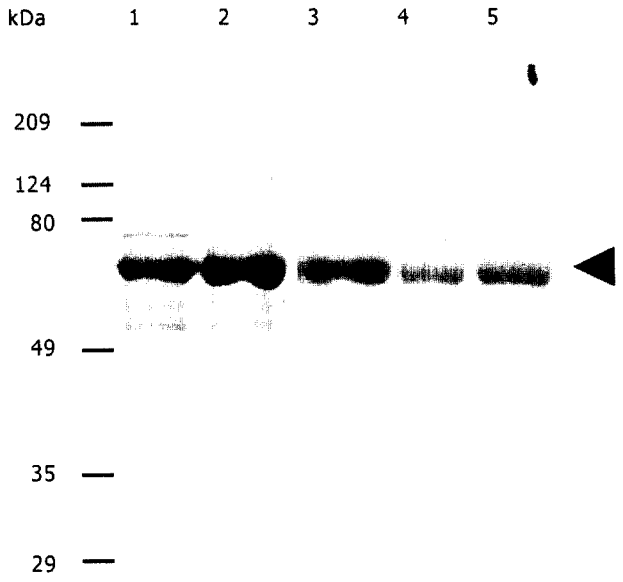

Figure 3. SDS-PAGE of isolated human serum albumin. Arrowhead indicates albumin. Lane 1, crude human serum; lane 2, serum albumin fraction in Blue-Sepharose chromatography; lane 3, albumin fraction in gel-filtration chromatography; lane 4, crude human urine of a patient; lane 5, urine albumin fraction in Blue-Sepharose chromatography.

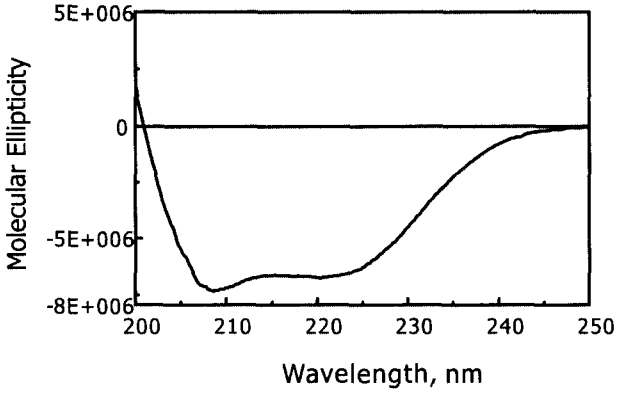

Figure 4. A typical far-UV spectra of human albumin. Circular dichroism measurements were made with purified albumin in 10 mM sodium phosphate buffer, pH 8.0, at a protein concentration of 350 g/350 l. Spectral bandwidth was 1 nm. The cuvette used had a light path length of 1 mm over 200250 nm. Scan speed was 50 nm/min. All scans wereas carried out five times, and averaged to mean values.

6. Monosaccharide composition analysis

Each 0.1 mg of protein was subjected to different conditions of .acid hydrolysis. For the analysis of neutral sugars, the protein was hydrolyzed in 2 M trifluoroacetic acid at 100℃ for 4 h. The hydrolyzate was evaporated to dryness using a SpeedVac (Savant Instruments, Holbrook, NY, USA), resuspended in distilled water, then subjected to high-pH anion exchange chromatography with pulsed amperometric detection (Bio-LC DX-300 Dionex, Sunnyvale, CA, USA). NaOH, (16 mM) at a flow rate of 1 mL/min, was used to separate monosaccharides on a CarboPac PA-1 column (Dionex, 4×250 mm) and a guard column (4×50 mm).

7. Statistical anaylsis

Data are expressed as meanSD. The *t*-test was applied for the physical and biochemical characteristics of patients with MCD and the controls at the onset of the study. However, the kurtosis of the data distributions of the conformations and glycation meant that they were not appropriate for parametric statistics. This was due to the fact that the coefficient of variation was relatively small: 2.8 in the serum of nephrotic syndrome patients for the α-helix, 2.4 in the serum of the control group for the β-turn, and 1.5 in the serum of the remission group for the random configuration of albumin (Table 2). These values indicate that the data were not distributed evenly and were concentrated too close to the mean values. Therefore, the comparisons of the compositions of the α-helix, β-conformation, β-turn, random configuration, and glycation were tested by nonparametric statistics, Mann-Whitney U test, or Wilcoxon signed-rank test for two groups, and by Kruskal-Wallis test for three or more groups. A probability value of $p<0.05$ was considered statistically significant.

RESULTS

1. Changes in glycation

The current study expresses glycation as the number of glucose molecules per albumin molecule. No significant difference in glycation was observed between the sera of control patients, of nephrotic syndrome patients (0.08±0.08 vs 0.10±0.05, p=0.79) and between the sera of those with nephrotic syndrome and those in remission (0.10±0.05 vs 0.09 ±0.06, p=0.85). The glycation of albumin in the urine of patients with nephrotic syndrome was 0.72±0.05. In all subjects, the molar ratio of glucose to protein was less than 0.6

Table 2. Composition of the α-helix, β-conformation, and β-turn of albumin in terms of secondary structure.

Group	α-helix	β-conformation	β-turn	random
Control	34.1±2.3	12.8±2.0	21.2±0.5	31.9±1.1
Relapse of MCD	33.2±0.9	14.7±3.2	20.6±1.3	31.8±0.8*
Remission of MCD	33.5±1.0	12.7±2.0	21.1±0.9	32.7±0.5*

Data are expressed as meanSD. *p<0.05 by ANOVA test. MCD, minimal change disease

±0.5, which meants that less than one glucose molecule was bound to an albumin molecule. Other neutral monosaccharides were detected in these albumin fractions, but this might be attributable to contaminants since there were few impurities in the albumin fraction isolated by Blue-Sepharose or gel-filtration chromatography (Figure 2). This suggests that urine albumin of the patients in this study was excreted independently of glycation.

2. Conformational changes in albumin

The composition of the α-helix, β-conformation, and β-turn of albumin in terms of secondary structure is summarized in Table 2. In the secondary structure of albumin, the α-helix, random configuration, β-turn, and β-conformation were observed in this order of frequency in all of the samples. There was no difference in the composition of the α-helix, β-conformation, and β-turn of albumin between the sera of the controls patients to of those with nephrotic syndrome.

Conformational change in serum albumin between nephrotic syndrome and remission patients : In this study, the proportion of the random configuration was calculated by subtracting the sum of the percentages of α-helix, β-conformation, and β-turn from 100%. The proportion of random configuration was slightly higher in the plasma albumin of relapsing patients than in the patients in remission (31.8± 0.8% vs 32.7±0.5%, p=0.049). However, there was no significant difference in the proportions of each of the α-helix, β-conformation, and β-turn in the plasma albumin between relapsers and those in remission. These observations imply that the α-helix, β-conformation, and β-turn components participate collectively with regards to changes in the random configuration, rather than one of either the α-helix, β-conformation, or β-turn playing the major role.

Conformational change of albumin between the serum and urine of nephrotic syndrome patients : The proportion of random configuration was lower in the albumin of the serum of nephrotic syndrome patients than in their urine (p=0.46, Wilcoxon signed-rank test). Conversely, there was no difference in the proportions of α-helix, β-conformation, and β-turn of albumin between their plasma and urine.

DISCUSSION

No difference was observed in both the conformational change and glycation of albumin of either before or after steroid replacement, regardless of the responses to it. A previous study reported that, in healthy humans, glycated albumin represents around 1.5% of total albumin[27]. In the current study, glycation at the molecular level was quantitatively expressed as molecules of glucose per molecule of albumin. Our results showed that the degree of glycation was negligible; only a very small proportion of albumin molecules were glycated and even those contained only a single molecule of glucose. These results suggest that the glycation of albumin is not a factor influencing the passage of albumin through the glomerulus barrier in patients with MCD. Moreover, the glycation process did not appear to be influenced by glucocorticoid replacement. Other neutral monosaccharides were detected in these albumin fractions, but they appeared to represent contamination since there were few impurities in the albumin fraction isolated by Blue-Sepharose or gel-filtration chromatography (Figure 2).

Albumin has long been known to be a highly helical molecule, and X-ray diffraction has revealed 67% of the residues of crystalline human serum albumin[28]. However, the presence of a α-helix, β-sheets and bend, and random coil can be predicted by several physical and sequence-related methods. Pearson[29] predicted the proportions of α-helix, β-turn, and β-sheet are 65%, 19%, and 10% in human serum albumin, respectively. In the current study, the proportions of α-helix, random configuration, β-turn, and β-conformation in the serum albumin of the healthy control group were 34.1%, 31.9%, 21.2%, and 12.8%, respectively. In this study, the proportions of the β-conformation, β-turn, and the random configuration were higher in the albumin of the serum than in the urine of patients with nephrotic syndrome. Two possible explanations for this observed difference are (1) the conformational characteristics of the circulating albumin are heterogeneous and some parts of albumin are selectively passed through the barrier in the glomerulus, and (2) a small degree of conformational transition occurs in the urinary space after the passage of the glomerular basement membrane. Further investigation into these possibilities is needed.

The proportion of random configuration was determined by subtracting the sum of the percentage composition of α-helix, β-conformation, and β-turn from 100%. This proportion was slightly higher in the serum albumin of patients with nephrotic syndrome than in the plasma of those in remission (32.7±0.5% vs 31.8±0.8%, p=0.049). This discrepancy implies that individual changes in the conformations of α-helix, β-conformation, and β-turn were not detectable by the statistical methods we applied, whereas their sum was.

The present study does not explain the mechanism underlying these conformational transitions. However, it is known that the albumin molecule is not in a static state; it is flexible and rapidly changing in shape[28]. The entire molecule tumbles in about 40 ns (based on the rotational diffusion coefficient)[28]. Its loop-link structure permits rapid expansion,

contraction, and flexion - some of this intrinsic, and some of this related to the binding of ligands[28]. The high exchangeability of hydrogen atoms is known to be a unique characteristic of albumin among nonenzymatic proteins[30, 31], and this probably represents a corollary of its loose structure and its propensity for binding many ligands. Like other proteins, components of albumin are also constantly moving on more rapid time scales. Gurd and Rothgeb[32] have reviewed these motions, and determined that reorientation of amino acid side chains occurs in 10^{-10}-10^{-11} s, and ionizations in 10^{-11} s. Hence, although albumin in solution can be considered as having a single shape overall, it is probably more realistic to regard it as an assembly of moving, resilient parts, that frequently change in conformation through the opening and closing of major crevices. Taking these characteristics of albumin and our results together, we speculate that the factors that act differently between the remission and relapse of albuminuria influence the pathogenesis of albuminuria in patients with MCD.

In conclusion, our results suggest that conformational changes of albumin, which is a complex of a α-helix, β-conformation, and β-turn, are a factor for albuminuria in patients with MCD. The glycation of albumin does not appear to influence the passage of albumin through the glomerulus barrier in patients with MCD.

ACKNOWLEDGEMENT

This work was partially supported by the National Research Laboratory Program (#M10104000270), Korea.

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