The Meaning of Serum Levels of Advanced Glycosylation End Products in Diabetic Nephropathy

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It has been reported that advanced glycosylation end products (AGEs) play an important role in the development of diabetic complications. To evaluate the relationship between serum AGEs and diabetic nephropathy, we measured serum AGE levels in diabetic patients with normoalbuminuria (N), microalbuminuria (M), overt proteinuria (O), and hemodialysis (HD), non diabetic patients with nephropathy, and age-matched control subjects using the enzyme-linked immunosorbent assay (ELISA). Urine AGE levels were also measured in these subjects except group HD. Serum AGE levels in diabetic patients were not significantly higher than those in the normal subjects. When we compared serum AGE levels among various stages of diabetic nephropathy, groups O and HD had significantly higher serum AGE levels than the other groups. Serum AGE levels in group HD were almost 6-fold higher than those in groups N and M. In contrast, there were no significant differences in urinary AGE levels among any diabetic groups. As for the variables that determine serum AGE levels in diabetic patients, there was no significant correlation between serum AGEs and fasting blood glucose, hemoglobin A_{1c} (HbA_{1c}), or duration of diabetes. In contrast, serum AGEs showed a strong correlation with serum creatinine and an inverse correlation with creatinine clearance. To evaluate the relationship between serum AGEs and oxidative stress in diabetic nephropathy, urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and serum malondialdehyde (MDA), which are biological markers of total oxidative stress in vivo, were also examined. Both urinary 8-OHdG and serum MDA levels were significantly higher in diabetic patients with proteinuria versus those without proteinuria. However, there was no significant correlation between serum AGEs and urinary 8-OHdG or serum MDA levels in diabetic patients. These results suggest that the accumulation of serum AGEs in diabetic nephropathy may be mainly due to decreased removal in the kidney rather than increased production by high glucose levels or oxidative stress.

ADVANCED GLYCOSYLATION end products (AGEs) are produced by a nonenzymatic reaction between proteins and physiologic sugars in vivo; they accumulate in tissues as a function of time and sugar concentration. These AGEs may contribute to the development of diabetic complications.[1-3] It has been reported that a highly significant correlation exists between an accumulation of AGEs on collagen and the severity of diabetic complications.[4-6] Immunochemical analyses with anti-AGE antibodies detected AGEs in in vivo tissues: aorta, lens crystallin, and renal cortex.[7-9] These results suggested that AGEs may play an important etiological role in the development of diabetic complications.

Since frequent measurements of AGE levels in tissue specimens are difficult in clinical practice, the use of AGEs as a disease parameter has been restricted. However, blood samples can be monitored. AGEs such as pentosidine and *N*ξ-(carboxymethyl)lysine (CML) have been traditionally quantified by high-performance liquid chromatography (HPLC) or gas chromatography–mass spectrometry, which are complicated processes. Then, enzyme-linked immunosorbent assays (ELISAs) using anti-AGE antibodies were established, although the AGE structures recognized with the assays remain unknown.[7,10,11] There have been various anti-AGE antibodies, so their detection of AGEs may be different. As for AGEs in blood samples, it has been reported that circulating AGEs are increased in the blood of diabetic patients, especially with end-stage renal disease.[7,12-18] These data have been reported using a radioreceptor assay,[12] an ELISA with anti-AGE antibodies,[7,13-16] HPLC,[17] and an AGE-specific fluorescence assay.[18]

In this study, to evaluate the relationship between serum AGEs and diabetic nephropathy, we determined serum and urinary AGE levels in diabetic patients with various stages of diabetic nephropathy and age-matched controls using the ELISA method, which was recently established.[16] It has been speculated that enhanced AGE formation in diabetic patients is closely linked not only to increased nonenzymatic glycation but also to increased oxidative stress. To determine the mechanism for increased serum AGE levels in diabetic nephropathy, therefore, we also measured the urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration, which was recently reported to be a sensitive biological marker of oxidative DNA damage and also of the total systemic oxidative stress in vivo.[19]

SUBJECTS AND METHODS

Patients

The subjects were 122 age-matched patients: 73 diabetic patients (25 undergoing hemodialysis and 48 not), 24 nondiabetic patients with nephropathy (19 undergoing hemodialysis and 5 not), and 25 normal patients. All subjects were without liver dysfunction or malignancy. The clinical characteristics of the subjects are shown in Table 1. The diabetic patients were subdivided into 4 groups, no proteinuria (N), microalbuminuria (M), overt proteinuria (O), and end-stage renal disease requiring hemodialysis (HD). Blood samples were collected at fasting except in patients undergoing dialysis, which were collected before the dialysis procedure. Urine samples were collected for 24 hours. Blood samples were analyzed for blood glucose, hemoglobin A_{1c} (HbA_{1c}), serum creatinine, cholesterol, malondialdehyde (MDA), and AGE.

AGE Measurement

Each blood and urine specimen was centrifuged at 3,000 rpm for 10 minutes. The serum sample was passed through an ultrafiltration device

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Submitted September 15, 1999; accepted January 28, 2000.

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0026-0495/00/4908-0022$10.00/0
doi:10.1053/meta.2000.7738

Table 1. Characteristics of the Study Subjects (mean ± SE)

Group	No. of Subjects (M/F)	Age (yr)	Duration of Diabetes (yr)	Serum Creatinine (mg/dL)	HbA_{1c} (%)	Serum Cholesterol (mg/dL)	Duration of HD (mo)
Controls	25 (18/7)	54.0 ± 1.9		0.70 ± 0.06			
Diabetic patients							
N	28 (19/9)	54.0 ± 1.8	7.3 ± 1.3	0.73 ± 0.04	9.5 ± 0.4	208.2 ± 10.7	
M	15 (8/7)	57.6 ± 2.5	13.9 ± 2.3	0.70 ± 0.06	10.0 ± 0.6	212.6 ± 9.1	
O	17 (13/4)	59.8 ± 3.1	20.4 ± 2.5	3.22 ± 0.46	8.0 ± 0.6	190.7 ± 18.8	
HD	21 (14/7)	59.2 ± 1.7	17.4 ± 1.5	9.96 ± 0.53	6.8 ± 0.2	163.2 ± 7.6	48.1 ± 11.3
Nondiabetic patients							
Non-HD	5 (2/3)	58.2 ± 5.9		6.33 ± 1.64		220.7 ± 52.4	
HD	19 (12/7)	52.3 ± 1.6		15.32 ± 1.88			121.3 ± 16.5

Abbreviations: N, normoalbuminuria; M, microalbuminuria; O, overt proteinuria; HD, end-stage renal disease requiring hemodialysis; M, male; F, female.

(Microcon 10; Amicon, Beverly, MA) to separate a high–molecular weight (>10 kd) protein fraction from a low–molecular weight fraction (≤10 kd). Serum and urine samples were stored at −80°C until analysis.

Serum and urine AGE levels were measured by an ELISA method using anti-AGE keyhole limpet hemocyanin.[16] This antibody did not cross-react with Amadori products. Amadori products in the serum samples were reduced by $NaBH_4$/50 mmol/L NaOH at a step in the pretreatment of the serum samples, and therefore, they were not detected by the assay. The antibody cross-reacted with CML and reacted with pentosidine slightly.[16] The intraassay coefficient of variation for this ELISA system was 4.8% to 10.2%, and the interassay value was 3.5% to 6.2%.[16] Results are expressed as arbitrary AGE units (1 mU AGE corresponds to 4 μg AGE-BSA standard).

Measurement of 8-OHdG in Urine Samples

The sample of well-mixed 24-hour urine collection was stored frozen at −80°C until analysis. Urine samples were centrifuged at 10,000× *g* for 10 minutes, and the supernatant was used for the determination of 8-OHdG by a competitive ELISA (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). The determination range was 0.64 to 2,000 ng/mL. The specificity of the monoclonal antibody N45.1 used in the competitive ELISA has been established.[20] Urinary 8-OHdG is expressed as the total amount excreted in 24 hours, or the urinary 8-OHdG to creatinine ratio.

Blood glucose was determined by the glucose oxidase–immobilized enzyme electrode method (Glucoroder-MK II; AT, Tokyo, Japan). Serum creatinine was analyzed by the alkaline picric acid method (Hitachi 7450E Automatic Analyzer; Hitachi Seisakusho, Tokyo, Japan), and cholesterol, by the enzymatic method (Hitachi Automatic Analyzer 7170). HbA_{1c} was analyzed by HPLC (Automatic Glycated Hemoglobin Analyzer HLC-723GHbIIIs; Tosoh, Tokyo, Japan), and the normal level was 5.1% ± 0.3% (mean ± SD). The serum MDA level was measured by the thiobarbituric acid (TBA) reaction method (MDA Test Wako; Wako Junyaku, Osaka, Japan), and the normal level was 1.8 to 4.7 mmol/mL.

Statistical Analysis

Statistical analysis was performed by ANOVA followed by Fisher's comparison test and linear regression using Statview J 4.5 software (Abacus Concepts, Berkeley, CA). Values are expressed as the mean ± SE. *P* values less than .05 are significant.

RESULTS

Serum AGE levels in diabetic patients without hemodialysis were 3.8 ± 0.5 mU/mL, not significantly higher versus the normal subjects (2.5 ± 0.1 mU/mL, $P = .1225$). Next, we evaluated the relationship between the stage of diabetic nephropathy and serum AGEs. Serum AGE levels in group HD (20.1 ± 1.7 mU/mL) were markedly higher than those in each of the other groups (N, 3.0 ± 0.1 mU/mL, $P < .0001$; M, 2.8 ± 0.1 mU/mL, $P < .0001$; O, 5.0 ± 0.8 mU/mL, $P < .0001$; and normal subjects, 2.6 ± 0.1 mU/mL, $P < .0001$). And serum AGE levels in group O were significantly higher than those in the normal subjects ($P = .0008$) (Fig 1A). Furthermore, we determined which serum AGEs in the high–molecular weight protein fraction or the low–molecular weight peptide fraction were increased. AGE levels in the protein fraction from group HD (18.6 ± 1.6 mU/mL, $P < .0001$) were significantly higher than those in any of the other groups (N, 1.9 ± 0.2 mU/mL,

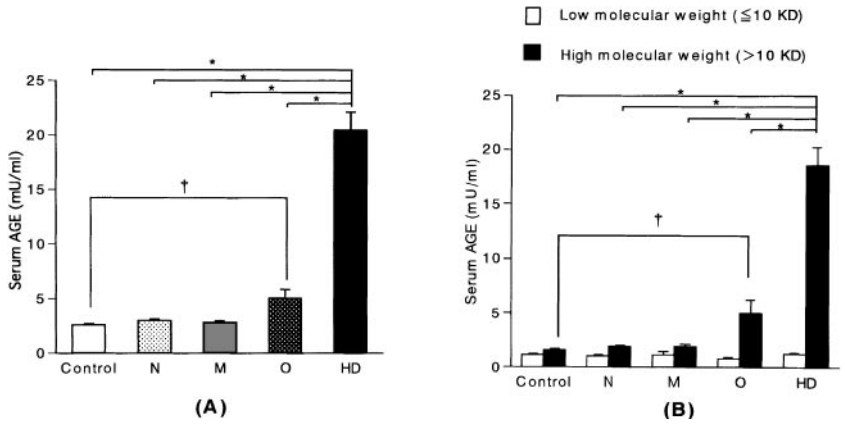

Fig 1. Serum AGE levels in patients with various stages of diabetic nephropathy. (A) Total AGEs, (B) AGEs in the low–molecular weight fraction and the high–molecular weight fraction. N, normoalbuminuria; M, microalbuminuria; O, overt proteinuria; HD, hemodialysis. Values are the mean ± SE. *$P <$ .0001, †$P <$ .001.

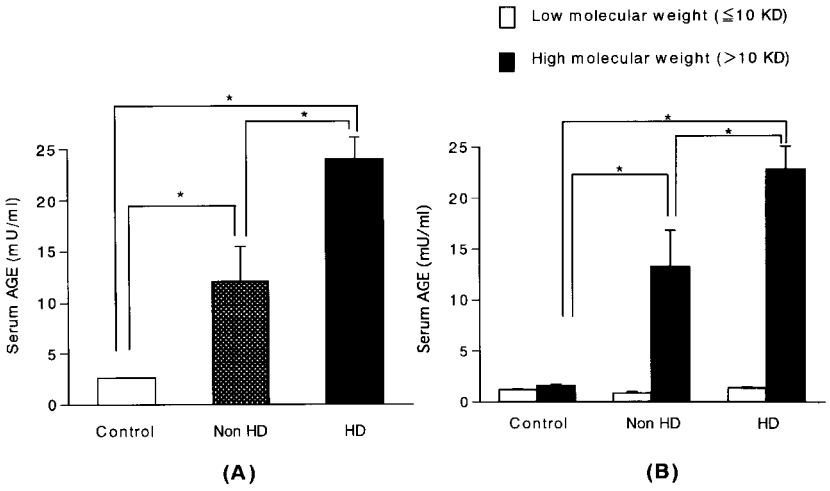

Fig 2. Serum AGE levels in patients with nondiabetic nephropathy. (A) Total AGEs, (B) AGEs in the low–molecular weight fraction and the high–molecular weight fraction. Values are the mean ± SE. *$P < .0001$.

$P < .0001$; M, 1.9 ± 0.2 mU/mL, $P < .0001$; O, 5.0 ± 1.3 mU/mL, $P < .0001$; and normal subjects, 1.6 ± 0.1 mU/mL, $P < .0001$). AGE levels in the protein fraction from group O were significantly higher than those in the normal subjects ($P = .0006$). In contrast, there were no significant differences in AGE levels in the peptide fraction among any diabetic groups (Fig 1B).

We also compared serum AGE levels in diabetic and nondiabetic nephropathy. Serum AGEs in nondiabetic patients undergoing hemodialysis (22.8 ± 2.3 mU/mL) were significantly higher than those in the nondiabetic patients with nephropathy without hemodialysis (13.3 ± 3.5 mU/mL, $P < .0001$). Serum AGE levels in both nondiabetic patients with nephropathy and those undergoing hemodialysis were higher than in the normal subjects (both $P < .0001$) (Fig 2). In addition, serum AGE levels in nondiabetic patients with hemodialysis were not significantly different from those in the diabetic patients with hemodialysis.

As for the urinary concentration of AGEs, there were no significant differences among any stages of diabetic nephropathy. The mean AGE levels in groups O, M, and N were 1.18 ± 0.18, 1.12 ± 0.17, and 1.53 ± 0.13 U/d, respectively (Fig 3).

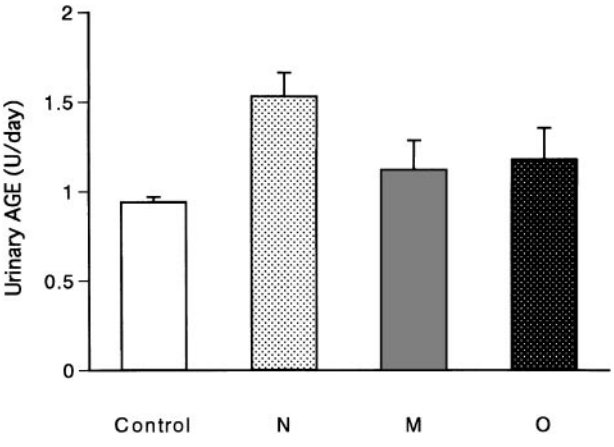

Fig 3. Urinary AGE levels in patients with various stages of diabetic nephropathy. N, normoalbuminuria; M, microalbuminuria; O, overt proteinuria; HD, hemodialysis. There were no significant differences among any stages of diabetic nephropathy. Values are the mean ± SE.

In the diabetic groups without hemodialysis, there was no correlation between serum AGEs and fasting blood glucose or HbA_{1c}. Serum AGE levels tended to correlate, but not significantly, with the duration of diabetes ($r = .253$, $P = .0557$). Serum AGEs in diabetic patients showed a strong positive correlation with serum creatinine ($r = .842$, $P < .0001$) and an inverse correlation with creatinine clearance (Fig 4).

Furthermore, to evaluate the relationship between serum AGE levels and oxidative stress in diabetic nephropathy, total 24-hour urinary 8-OHdG excretion and serum MDA levels were determined. The total 24-hour urinary 8-OHdG excretion in group O was significantly higher versus the control subjects and group N (32.4 ± 5.5 *v* 14.3 ± 2.4 and 15.4 ± 1.9 μg/d, $P < .05$, respectively) (Fig 5A). The urinary 8-OHdG to creatinine ratio was also significantly higher in group O versus group N (35.9 ± 8.2 *v* 17.9 ± 1.4 μg/g Cr, $P < .05$) (Fig 5B). However, there was no significant correlation between serum AGEs and the total 24-hour urinary 8-OHdG excretion (8-OHdG to creatinine ratio) or serum MDA levels in diabetic patients (Fig 6).

DISCUSSION

Intensive insulin treatment with an improvement of glycemic control effectively delays the onset and slows the progression of diabetic nephropathy in patients with type 1 diabetes[21,22] and experimental animals.[23-25] Hyperglycemia is therefore the main determinant of the initiation and progression of diabetic nephropathy. Evidence from animal experiments and clinical studies has suggested that the glomerular injury associated with hyperglycemia in diabetes mellitus may be largely mediated by endogenously formed AGEs. An accumulation of AGEs in the renal tissue of diabetic animals[1,9] and humans[26] has been reported. Immunohistochemically, AGEs were detected in an expanded mesangial matrix, especially in nodular lesions from patients with diabetic nephropathy.[26] However, it is difficult to use the renal tissue AGE level as a clinical parameter. In this study, we evaluated the relationship between serum AGEs and various stages of diabetic nephropathy. The results show that increased serum AGE levels were found in diabetic patients with overt proteinuria and those undergoing hemodialysis.

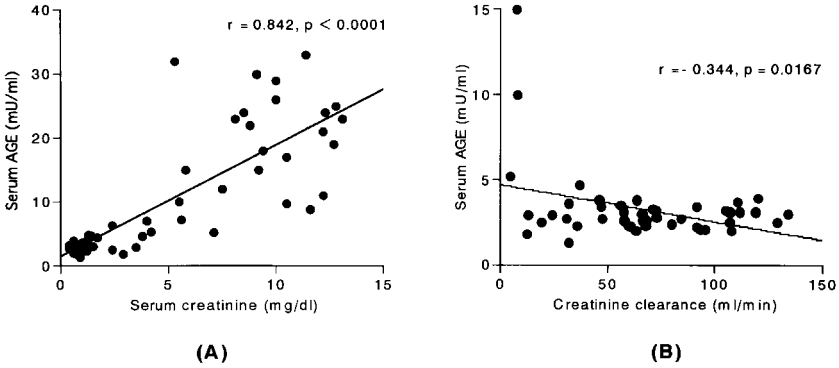

Fig 4. Correlation between serum AGE levels and (A) serum creatinine or (B) creatinine clearance in diabetic patients.

Increased serum AGEs showed a strong positive correlation with serum creatinine and an inverse correlation with creatinine clearance. In contrast, there were no significant differences in urinary AGE levels among any stages of diabetic nephropathy. These results imply that increased serum AGEs might be due to a decreased clearance of AGEs in the kidney rather than increased production. Accumulated serum AGEs in both patients with overt proteinuria and those undergoing hemodialysis were high–molecular weight substances. This finding is inconsistent with previous reports showing that 80% of serum AGEs in diabetic patients with renal failure are low–molecular weight peptides.[12-14] The reason for this discrepancy is unclear. AGEs are currently believed to include diverse structures. The AGEs detected in the present assay might be different from those reported previously. The antibody that we used cross-reacted with CML and reacted with pentosidine slightly, whereas it did not cross-react with Amadori products.[16]

As for the factors except renal function that determine serum AGE levels, the present results show that serum AGE levels were not significantly correlated with HbA_{1c} or the duration of diabetes. The detected AGEs in the present assay may be mainly CML.[16] CML was identified as a product formed upon oxidative cleavage of fructose lysine[27] and also by reaction with autoxidative products of ascorbate[28] and glucose.[29] Therefore, CML was identified as a product of the combined process of not only glycation but also oxidation. Miyata et al[30] suggested that the generation of AGEs in uremia was increased by high oxidative stress. The increased AGE levels might be partly due to this generation of AGEs by oxidative stress. The present results show that the total amount of urinary 8-OHdG excretion, a sensitive biomarker of oxidative DNA damage and also of the total systemic oxidative stress in vivo, was increased in diabetic patients with overt proteinuria. This is in agreement with a recent report.[31] These results support a role of oxidative stress in the pathogenesis of diabetic nephropathy. However, no significant correlations between serum AGE levels and urinary 8-OHdG excretion or serum MDA levels were found in diabetic patients. Taken together, these findings support the notion that increased serum AGEs in patients with nephropathy are mainly due to decreased clearance rather than increased production by glycation or oxidative stress.

One report showed that serum AGE levels predicted the progression of early morphological kidney damage in patients with NIDDM.[32] Others also showed the usefulness of serum AGE levels for the evaluation and follow-up of diabetic nephropathy.[16,33] In contrast, the present results imply that serum AGEs might not be useful for the clinical parameter of diabetic nephropathy, since serum AGE levels appeared to be determined by renal function alone. This notion is further supported by the evidence that serum AGE levels were increased in nondiabetic patients with renal failure and undergoing HD to the same extent as in diabetic patients. Although serum AGEs may not be involved in the pathogenesis of diabetic nephropathy, it is possible that AGEs formed in renal tissue may contribute to the development of diabetic nephropathy. Numerous studies have shown that aminoguanidine, an AGE inhibitor, prevents glomerular lesions and albuminuria in several experimental and genetic models of diabetes. The serum AGE level may be a useful marker for the therapeutic effect of such agents. It is also possible that specific serum AGEs might

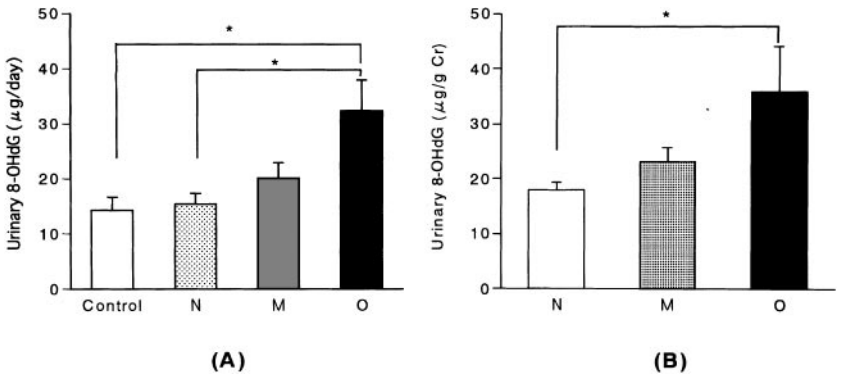

Fig 5. (A) Total amount of 24-hour urinary excretion of 8-OHdG and (B) urinary 8-OHdG/creatinine ratio in patients with various stages of diabetic nephropathy. N, normoalbuminuria; M, microalbuminuria; O, overt proteinuria; HD, hemodialysis. Results are the mean ± SE. *$P < .05$.

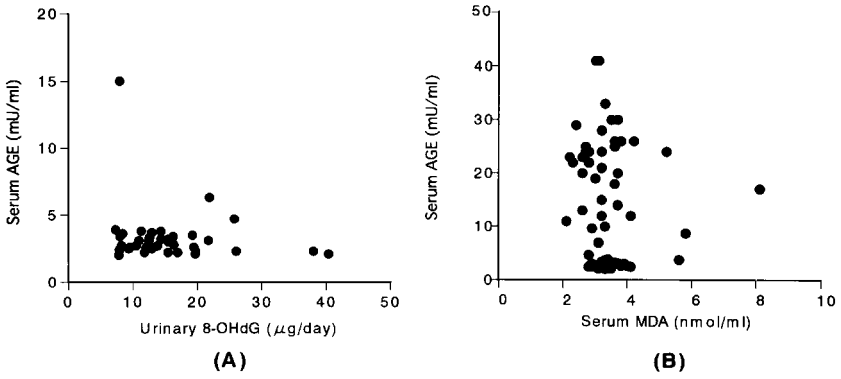

Fig 6. Correlation between serum AGE levels and (A) total 24-hour urinary excretion of 8-OHdG or (B) serum MDA in diabetic patients.

be involved in the pathogenesis of diabetic nephropathy, since AGEs include diverse structures. Further studies may be needed to detect specific serum AGEs that are involved in the pathogenesis of diabetic nephropathy.

In conclusion, increased serum AGE levels in patients with nephropathy may be mainly due to decreased clearance in the kidney rather than increased production by glycation or oxidative stress.

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