

Glucoselysine is derived from fructose and accumulates in the eye lens of diabetic rats

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Prolonged hyperglycemia generates advanced glycation endproducts (AGEs), which are believed to be involved in the pathogenesis of diabetic complications. In the present study, we developed a polyclonal antibody against fructose-modified proteins (Fru-P antibody) and identified its epitope as glucoselysine (GL) by NMR and LC-electrospray ionization (ESI)- quadrupole TOF (QTOF) analyses and evaluated its potential role in diabetes sequelae. Although the molecular weight of GL was identical to that of fructoselysine (FL), GL was distinguishable from FL because GL was resistant to acid hydrolysis, which converted all of the FLs to furosine. We also detected GL in vitro when reduced BSA was incubated with fructose for 1 day. However, when we incubated reduced BSA with glucose, galactose, or mannose for 14 days, we did not detect GL, suggesting that GL is dominantly generated from fructose. LC-ESI-MS/MS experiments with synthesized $[{}^{13}C_6]GL$ indicated that the GL levels in the rat eye lens time-dependently increase after streptozotocininduced diabetes. We observed a 31.3-fold increase in GL 8 weeks after the induction compared with nondiabetic rats, and $N\epsilon$ -(carboxymethyl)lysine and furosine increased by 1.7- and 21.5-fold, respectively, under the same condition. In contrast, sorbitol in the lens levelled off at 2 weeks after diabetes induction. We conclude that GL may be a useful biological marker to monitor and elucidate the mechanism of protein degeneration during progression of diabetes.

Reducing sugars and carbonyl compounds, such as glucose and methylglyoxal (MG),² nonenzymatically react with the

amino and thiol groups of amino acids to form advanced glycation end-products (AGEs) through the Schiff base and Amadori products of the Maillard reaction (1). Accordingly, AGEs are used as markers for carbohydrate metabolism *in vivo*. For instance, hemoglobin A1c (HbA1c) (2), glycoalbumin (3, 4), and fructoselysine (FL) (5) are well-known glycated proteins and lysed by glucose, serving as clinical markers for the diagnosis of diabetes. The modification of proteins by glycation and AGEs denatures the protein structures, thereby reducing enzymatic activity (6–9). Recent studies have demonstrated that AGEs accumulate in tissues during aging (10–12) as well as under pathological conditions such as diabetic complications, including kidney failure (13, 14), retinopathy (15–17), neuropathy (18), and atherosclerosis (19, 20).

We previously reported that $N\epsilon$ -(carboxymethyl)lysine (CML), a major antigenic AGE, was generated by oxidation with hydroxyl radicals (21), hypochloric acid (22), and peroxynitrite (23), demonstrating its role as an oxidative marker. Thornalley *et al.* (14) demonstrated that the level of the MGderived AGE, N^{δ} -(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine is increased in the plasma of patients with kidney failure. We also previously reported that a glycolaldehyde (GA)-derived AGE, GA-pyridine accumulates in human atherosclerosis lesions (19). CML and N^{ϵ} -(carboxyethyl)lysine (CEL) are known to accumulate in the human lens with aging (10–11).

These reports demonstrated the variation in the AGEs generated in different tissues and under different pathological conditions. Therefore, we hypothesized that levels of AGEs or glycated products could be a valuable marker for metabolic abnormalities.

Although extensively researched, we focused on fructosederived AGEs that accumulate in the lens. We previously developed a polyclonal antibody against fructose-modified protein and showed that the epitope of the antibody accumulates in the lens of diabetic rats and the content correlated with the amount of sorbitol in the lens (24). Because activation of the polyol



The authors declare that they have no conflicts of interest with the contents of this article.

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² The abbreviations used are: MG, methylglyoxal; ACN, acetonitrile; AGE, advanced glycation end-product; RdBSA, reduced bovine serum albumin; LC-ESI, liquid chromatography-electrospray ionization; LC-ESI-QTOF, LC-ESI-quadrupole-time of flight; BCA, bicinchoninic acid; Cbz, carboxybenzyl; CML, *Nε*-(carboxymethyl)lysine; FA, formic acid; FL, fructoselysine; Fru-P, fructose-modified protein; GL, glucoselysine; HbA1c, hemoglobin A1c; KLH, keyhole limpet hemocyanin; MRM, multiple reaction monitoring; TBS,

Tris-buffered saline; GA, glycolaldehyde; STZ, streptozotocin; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation.

Weeks after STZ injection	0	1	2	3	4	6	8	
Body weight								
Normal $(n = 6)$	157.2 ± 5.2	201.7 ± 7.4	231.7 ± 9.3	277.5 ± 12.3	307.7 ± 15.0	362.2 ± 20.9	401.0 ± 24.9	
$DM^a (n = 6)$	157.1 ± 5.8	183.3 ± 13.3	201.9 ± 19.2	235.7 ± 19.0	254.0 ± 21.2	257.0 ± 44.5	281.0 ± 46.9	
Blood glucose								
Normal $(n = 6)$	87.3 ± 17.0	63.1 ± 27.4	98.3 ± 7.6	89.7 ± 20.3	105.7 ± 9.1	105.8 ± 8.8	211.0 ± 38.4	
DM(n=6)	99.1 ± 35.3	321.3 ± 18.7	384.1 ± 39.7	380.8 ± 84.6	461.5 ± 38.5	496.4 ± 38.2	537.1 ± 68.6	
HbA1c								
Normal $(n = 6)$	ND^{b}	3.1 ± 0.1	ND	ND	ND	ND	3.7 ± 0.1	
DM (n = 6)	ND	4.5 ± 0.2	5.8 ± 0.3	ND	7.5 ± 0.2	8.6 ± 0.8	9.2 ± 0.7	

Table 1	
Biochemical	parameters of rats after the induction of diabetes

^{*a*} DM, diabetic group.

^{*b*} ND, not determined.

pathway is enhanced under a hyperglycemic condition and is known to be associated with several diabetic complications, such as retinopathy, neuropathy, and kidney failure (25), the accurate measurement of fructose-derived AGEs may clarify the mechanism of the development and progression of such complications. However, the epitope structure of the polyclonal antibody has not been identified since this first report more than 20 years ago (24).

In the present study, we prepared a new antibody by a method different from a previously reported one designated as Fru-P antibody. Then we evaluated reactivity of its antibody with lens proteins of streptozotocin (STZ)-induced diabetic rats. We also compared reactivity of CML antibody in the same rats. Furthermore, the epitope structure of Fru-P antibody was identified and a system was developed for its quantification with LC-ESI-QTOF and LC-ESI-MS/MS to investigate its relationship to diabetes.

Results

Reactivity of Fru-P antibody with rat lens proteins

Type 1 diabetes was induced by STZ injection in Wistar rats, and the change in GL in the lens proteins was compared with other biological parameters. As shown in Table 1, the body weight of normal rats showed a steep increase in a time-dependent manner, whereas body weight of diabetic rats increased more gradually, with \sim 70.1% lower body weight than normal rats after 8 weeks. The fasting blood glucose levels of diabetic rats dramatically increased after the induction of diabetes, and gradually increased during the feeding period. In contrast, the blood glucose level of the normal rats did not change during the feeding period except at 8 weeks (Table 1). Furthermore, the levels of HbA1c increased during the feeding period in the diabetic rats; however, it did not change even at 8 weeks (Table 1) in normal rats. Western blotting showed that the levels of the epitope of the Fru-P antibody in the rat lens dramatically increased by the induction of diabetes (Fig. 1A), whereas the CML level was hardly affected by diabetes onset (Fig. 1D). The results of densitometric analysis of the bands detected by Western blotting demonstrated that the epitope of Fru-P antibody and CML increased 4.3- (Fig. 1B) and 1.1-fold (Fig. 1E), respectively, following the induction of diabetes. The residual proteins in the polyacrylamide gel, stained by Coomassie Brilliant Blue as a loading control, showed no difference (Fig. 1, C and F). Furthermore, the levels of AGEs measured by ELISA also demonstrated that the epitope structure of the Fru-P antibody and



Identification of the epitope structure of Fru-P antibody

To analyze the epitope structure of the Fru-P antibody, the antibody reactivity was measured by noncompetitive ELISA. As shown in Fig. 2*A*, Fru-P antibody reacted with fructose-modified BSA (Fru-BSA) and keyhole limpet hemocyanin (KLH) in a dose-dependent manner, whereas the reactivity to BSA modified with other aldehydes and native BSA was negligible. After incubation of fructose along with the basic amino acids, lysine and arginine, which are considered to be preferentially modified by aldehydes (26), the antibody was found to react only with fructose-modified acetyl-lysine in a dose-dependent manner (Fig. 2*B*), suggesting that its epitope structure was generated from lysine.

Carboxybenzyl (Cbz)-lysine was used to produce the epitope structure and the eluent was monitored with a UV detector at 270 nm, which is a characteristic of the Cbz group (first-step purification). Cbz-lysine was eluted at a retention time of 26-27 min, and the reaction mixture of Cbz-lysine and fructose generated several peaks, four of which were isolated (Fig. 2C). By competitive ELISAs, the reactivity of Fru-P antibody to coated Fru-BSA showed significant competition with Fru-BSA (as a positive control) and fraction 2 (Fig. 2D). Because fraction 2 contained at least two peaks, the reaction mixture was analyzed with a different elution program to improve the separation efficiency. As shown in Fig. 2E, Cbz-lysine was eluted at a retention time of 40-42 min. Fractions 2-1 and 2-2 were isolated and their reactivities were measured against the antibody. As a result, the reaction of Fru-P antibody to coated Fru-BSA was competed only by fraction 2-1 and fraction 2 (as a positive control) (Fig. 2F), demonstrating that fraction 2-1 is the epitope of Fru-P antibody.

NMR structural analysis

NMR analysis was conducted to determine the structure of fraction 2-1, formed from the condensation of Cbz-lysine and fructose. The one-dimensional ¹H NMR spectrum showed the presence of two anomeric signals at 5.53 and 5.03 ppm (Fig. 3*A*), suggesting that the sugar is in an equilibrium between α and β hemiacetal forms. The coupling constants of each anomeric signal were 3.3 and 8.4 Hz, respectively, suggesting that the sugar is a glucose-like and not a mannose-type configuration. The NMR signals were assigned by a series of one- and two-





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Figure 1. Reactivity of Fru-P antibody or CML antibody to the rat lens proteins after the induction of diabetes. Lens proteins (10 μ g/lane) were applied to 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The proteins bound to the membrane were detected by Western blot using Fru-P antibody (*A*) or CML antibody (*D*) and were quantified by densitometric analysis (*B* and *E*). Residual proteins on the polyacrylamide gel after blotting were stained by Coomassie Brilliant Blue as a loading control (*C* and *F*). Each well of a 96-well immunoplate was coated with 10 μ g/ml of lens protein, and the reactivity of 0.5 μ g/ml of Fru-P antibody (*G*) or CML antibody (*H*) were visualized by horseradish peroxidase-conjugated anti-rabbit IgG antibody and 1,2-phenylenediamine dihydrochloride as described under "Experimental procedures." *Nor*, normal at 8 weeks (*n* = 3); DM, diabetes at 8 weeks (*n* = 3). The data are presented as mean \pm S.D. #, *p* < 0.001 *versus* normal at 8 weeks (Student's *t* test).

dimensional experiments and the assignments are shown in Fig. 3*B*. To identify the linkage between the sugar and lysine, ¹H-¹³C HMBC and ¹H-¹⁵N HMBC spectra were collected (Fig. 3, *C* and *D*). Several HMBC peaks were successfully observed between sugar and the lysine side chain. Taken together, it was concluded that the ϵ -amino group of the lysine side chain is connected to the C2 position of glucose in the Fru-P antibody epitope (Fig. 3*E*). Therefore, the epitope of Fru-P antibody is also referred to hereafter as glucoselysine (GL).

Structural analysis by LC-ESI-QTOF

To confirm the structure analysis by NMR, the epitope structure was also analyzed by ESI-Q-TOF-MS/MS. An ion peak at m/z 443.2025 [M + H]⁺ for fraction 2-1 was estimated as $C_{20}H_{30}N_2O_9$ (m/z 443.2024) (Fig. 4A). After the Cbz group was deprotected by catalytic reduction using palladium carbon, a peak at m/z 309.1656 ($C_{12}H_{25}N_2O_7$) (Fig. 4B) corresponded to a similar mass-charge ratio of FL and GL. The fragment ions detected at m/z 309.1656 by MS/MS are shown in Fig. 4C and Table 2.

Chemical properties of GL and FL

Because GL and FL, generated from fructose and glucose, respectively, have the same m/z (C₁₂H₂₅N₂O₇) and elemental

composition, the chemical properties of these compounds were compared. As shown in Fig. 5A, GL was stable under acid hydrolysis against 6 M HCl at 100 °C for 48 h, whereas all of the FL was degraded within 18 h under the same condition. Furthermore, FL was degraded by acid hydrolysis in a time-dependent manner up to 4 h, and furosine was generated by 1 h incubation with HCl and a complete conversion was seen as early as 4 h (Fig. 5B). To compare the yields of FL and GL, they were incubated with BSA in the presence of fructose, glucose, galactose, or mannose; reduced BSA (RdBSA) was used to exclude the GL present in native BSA. The level of GL increased in a time-dependent manner under incubation with fructose, but not with glucose, galactose, or mannose, demonstrating that GL was preferentially generated from fructose (Fig. 5, C and E). In contrast, the level of furosine, the hydrolysate of FL, increased in a time-dependent manner when RdBSA was incubated with glucose, whereas a small amount of furosine was generated from fructose (Fig. 5D), suggesting that FL was predominantly generated from glucose, galactose, and mannose (Fig. 5F). To clarify the stability of GL under oxidative stress, GL was exposed to hydroxyl radicals because CML is generated by the oxidation of FL. As shown in Fig. 5G, CML was generated by exposure of FL to hydroxyl radicals from iron (II) chloride with hydrogen per-



Identification of glucoselysine in diabetic lens



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Figure 2. Isolation of the epitope structure of the Fru-P antibody. Amino acids producing the epitope were analyzed and the epitope structure was isolated by HPLC. *A*, each well of a 96-well immunoplate was coated with modified proteins, and the reactivity of Fru-P antibody (0.5 μ g/ml) was visualized by horseradish peroxidase-conjugated anti-rabbit IgG antibody and 1,2-phenylenediamine dihydrochloride as described under "Experimental procedures." *B*, Fru-BSA (0.01 μ g/ml) was coated on the immunoplate and the blocked with gelatin. Fifty microliters of each competitor was added in the presence of the same volume of Fru-P antibody (0.5 μ g/ml). The antibody bound to the well was visualized as described above. *C*, 100 μ l of fructose-modified Cbz-lysine (50 mM) was applied to first-step HPLC and separated into four fractions (*Fr.* 1–4). *D*, the reactivity of Fru-P antibody with the four isolated fractions was measured by competitive ELISA. *E*, *Fr.* 2 obtained from first-step HPLC was further applied to second-step HPLC. *F*, the reactivity of Fru-P antibody with Fr. 2-1 and 2-2 isolated from the second-step HPLC system was measured by competitive ELISA.

oxide. In contrast, the CML level was below the detectable limit when GL was exposed to hydroxyl radicals under the same condition. These data demonstrated that GL does not generate CML under oxidative conditions.

Quantification of sorbitol, furosine, CML, and GL in the rat lens by LC-ESI-QTOF or LC-ESI-MS/MS

The levels of sorbitol, furosine, CML, and GL in lens of diabetic and normal rats were analyzed by LC-ESI-QTOF or LC-ESI-MS/MS with an internal standard. Measurement of the fructose level in lens was not very stable probably because it rapidly reacts with proteins to form a protein-adduct. Therefore, we measured sorbitol, upstream of the formation of fructose in the polyol pathway, by LC-ESI-QTOF. The level of sorbitol reached a maximum and levelled off at 2 weeks after the induction of diabetes (Fig. 6A). As shown in Fig. 7A, the quantitative ions of GL and [$^{13}C_6$]GL were detected by LC-ESI-MS/MS. The levels of GL significantly increased in a time-dependent manner (Fig. 7B). Specifically, the levels of furosine and CML increased by 21.5- and 1.7-fold, respectively, at 8 weeks after the induction of diabetes, whereas the level of GL

increased by 31.3-fold after 8 weeks (Figs. 6, *B* and *C*, and 7, *B* and *C*). Although GL was detectable in rat kidneys, there was no statistical significance between the normal and diabetic groups $(0.341 \pm 0.203 \text{ mmol/mol of lysine} \text{ versus } 0.402 \pm 0.182 \text{ mmol/mol of lysine}, mean \pm \text{ S.D.}, n = 6 \text{ each}$).

Discussion

The detailed pathogenic mechanisms and factors related to diabetic complications remain elusive; however, the AGEs have recently emerged as a strong candidate mediating these effects (25). Although several AGEs structures have been identified in biological samples, CML is the most measured AGE in the world. For instance, CML accumulates with progression of diabetes (27), atherosclerosis (19), and diabetic complications, such as nephropathy (28) and retinopathy (17). The result of Western blotting and ELISA revealed that the Fru-P antibody showed higher reactivity with diabetic rat lens than anti-CML antibody. It is likely, therefore, that the epitope of the Fru-P antibody contributed to the modification of lens proteins under hyperglycemia. Although an antibody against fructose-modified proteins was first reported by one of our groups in 1998 (24,



Figure 3. NMR analysis of the epitope structure recognized by the Fru-P antibody. NMR spectra of fraction 2-1 shown for (*A*) 1D ¹H, (*B*) 2D ¹H-¹³C HSQC, (*C*) 2D ¹H-¹³C HMBC, and (*D*) 2D ¹H-¹⁵N HMBC. The assignments are labeled for each peak. *E*, chemical structure of glucoselysine.

29), the epitope structure had not been identified until now. Furthermore, although the level of AGEs is a common clinical biomarker of diabetes and related complications, the detection of AGEs in physiological samples with immunological methods is affected by pretreatment of heating (30), alkaline treatment (31), and the presence of autoantibodies against AGEs (32). Therefore, identification of each AGE structure and precise quantification technology are required to evaluate the biological significance of AGEs, which is a limitation for clinical laboratories.

Here, we demonstrated that Fru-P antibody recognizes fructose-modified KLH and BSA, but not their native proteins. Furthermore, this antibody did not recognize CML, a major antigenic AGE (33), or the proteins modified by MG and GA, demonstrating that the epitope structure of the antibody differs from that of AGEs identified to date. Detection of GL by Fru-P antibody may be affected by amino acid sequence and the 3-D structure around the GL because the molecular mass of the side residue on GL is merely 162 Da (whole molecular mass of GL including lysine is 308 Da).

Heyns *et al.* (34) proposed that fructose forms lysine-adduct and named it as GL, but did not confirm the structure by instrumental analysis. Furthermore, Kawasaki *et al.* (24) demonstrated that the reactivity of Fru-P antibody to the diabetic rat lens is positively correlated with sorbitol levels. Taken together, these reports suggested that fructose-modified proteins accu-





Figure 4. Structural analysis by LC-ESI-QTOF. *A*, ESI-QTOF analysis of fraction 2-1 showing an ion peak at m/z 443.2024 [M + H]⁺ calculated as C₂₀H₃₀N₂O₉. *B*, ESI-QTOF analysis of de-protected fraction 2-1 showing an ion peak at m/z 309.1656 [M + H]⁺ calculated as C₁₂H₂₅N₂O₇. *C*, MS/MS analysis of the de-protected fraction 2-1 of m/z 309.1656 [M + H]⁺ detected fragment ions are indicated in Table 2.

Table 2 Identification of glucoselysine fragment ion formulas by LC-ESI-QTOF

Expected m/z and formula	Detected m/z	Difference of m/z
84.0808 (C ₅ H ₁₀ N)	84.0799	-0.0009
$130.0863 (C_6 H_{12} NO_2)$	130.0844	-0.0019
210.1125 (C ₁₁ H ₁₆ NO ₃)	210.1081	-0.0044
225.1234 (C ₁₁ H ₁₇ N ₂ O ₃)	225.1191	-0.0043
255.1339 (C ₁₂ H ₁₉ N ₂ O ₄)	255.1271	-0.0068
273.1445 (C ₁₂ H ₂₁ N ₂ O ₅)	273.1415	-0.0030

mulate in the lens in accordance with progression of the polyol pathway.

Here, we showed that FL is converted into furosine by acid hydrolysis, whereas GL is stable under the same conditions. Although the FL content is typically estimated based on furosine levels after acid hydrolysis (data not shown), our previous experiment showed that the parent ion of FL remained detectable in biological samples even after acid hydrolysis for 18 h. However, because all of the purified FL was converted into furosine after acid hydrolysis for 4 h, it is likely that the level detected at m/z 309.1656, corresponding to the parent ion of FL, in physiological samples after acid hydrolysis reflected the presence of GL.

The parent ion of GL was also detected in commercial BSA after acid hydrolysis (data not shown), suggesting that GL may be generated in bovine serum. To account for the potential presence of endogenous GL and FL in biological samples, BSA was reduced with sodium tetrahydroborate before incubation with several kinds of carbohydrates to clarify the precursors for GL and FL. The results showed that GL was predominantly

generated from fructose, whereas FL was generated from mannitol, galactose, and glucose. Therefore, GL has potential to become a useful marker for protein modifications by fructose. FL is an Amadori rearrangement product, which is oxidized to form CML (21). In contrast, GL was not generated from CML after exposure to hydroxyl radicals, demonstrating its suitability for the clinical detection of AGEs. Because blood glucose levels sometimes transiently rise when animals get excited, levels of FL, measured as furosine in lens (Fig. 6*B*), and hemoglobin A1c (Table 1) were also determined to evaluate the variation of glucose level in the present study.

Nakayama *et al.* (35) and Turk *et al.* (36) demonstrated that the level of AGEs measured by the fluorescence intensity of lens proteins in normal rats did not change between 6 and 14 weeks of age. In contrast, the level of GL determined by LC-ESI-MS/MS showed a significant increase in the lens of normal rats by 8 weeks, suggesting that GL may increase as part of the aging process. Because the present study focused on identification of the structure generated from fructose, the presence of the structure *in vivo*, and its increase by the pathogenesis of diabetes in rat lens proteins, further study is required to clarify whether GL increases in accordance with normal aging.

Kawasaki *et al.* (24) demonstrated that the reactivity of Fru-P antibody to rat lens proteins increased after the induction of diabetes for 8 weeks. Surprisingly, in the present study, the reactivity of Fru-P antibody to the rat lens and the GL content measured by LC-ESI-MS/MS both increased 1 week after the induction of diabetes. Furthermore, the levels of GL, FL, and CML increased by 31.3-, 21.5-, and 1.7-fold, respectively, 8





Figure 5. Chemical properties of glucoselysine (*GL*) **and fructoselysine** (*FL*). The stability of GL (*A*) and FL (*B*) and formation of furosine under acid hydrolysis under 6 \bowtie HCl at 100 °C for 48 h were measured by LC-ESI-QTOF. The formation of GL (*C*) and furosine (*D*) during incubation of reduced-BSA (RdBSA) with fructose (*Fru*) or glucose (*Glu*) at 37 °C for up to 14 days. Comparison of GL (*E*) and furosine (*F*) formation during incubation of RdBSA with Fru, Glu, galactose (*Gal*), and mannose (*Man*) at 37 °C for 14 days. *G*, GL and FL were incubated in 50 mM sodium phosphate buffer (pH 7.2) in the presence or absence of FeCl₂ (0.4 mM) and H₂O₂ (0.1 mM) at 37 °C for 1 h, and CML formation was determined by LC-ESI-QTOF. The data are presented as mean \pm S.D. (*n* = 3). #, *p* < 0.001 *versus* control or Fru (Bonferroni test).

weeks after the induction of diabetes. There has been no previous report of an AGE structure increasing by ~20-fold within 8 weeks after the onset of diabetes. These results demonstrated that GL in the lens accumulates in accordance with the progression of the polyol pathway; the increasing rate was higher than FL and CML. Therefore, GL accumulation reflects the degeneration of lens proteins by fructose at an early stage. However, the level of GL in the rat kidney did not increase by the induction of diabetes. We have previously reported that the total 24-h urinary protein and albumin concentrations at 27 weeks were approximately five and six times higher, respectively, in diabetic rats compared with nondiabetic rats (27). Because rats were maintained for only 8 weeks after the induction of diabetes in the present study, kidney function may have been still uncompromised and GL levels in the kidneys did not alter significantly.

In the previous report, crystallin protein in the lens was shown to be denatured by MG, ribose, galactose, or fructose inducing aggregation, which is one of the causes of cataract. It has been reported that these aldehyde or hexoses form MG-H1, CEL, or CML (37, 38) suggesting that AGEs reflect the progression of cataract. However, these AGEs in the lens did not correlate with the development of cataract and they cannot be predictive markers of cataract. In this study, we focused on the association between GL accumulated in the rat lens and progression of diabetes, so the association with diabetic complications was not evaluated. Because GL shows a distinctly higher rate of increase than other AGEs, it may increase with the onset and progression of diabetic complications such as cataract. To clarify the possibility of using GL as a marker for polyol-related diabetic complications, further study is required to investigate its association with various durations of diabetes and related complications.

Experimental procedures

Sample preparation

GA-modified BSA and MG-modified BSA were prepared by incubating 2 mg/ml of BSA (1.5 mM lysine residues; initial fraction by heat shock, minimum 98%; Sigma) with 30 mM GA or MG (Sigma), respectively, at 37 °C for 7 days in PBS (26). To prepare the Fru-BSA, 10 mg/ml of BSA was mixed with 0.37 M D-fructose (Kanto Chemical Co., Inc., Tokyo, Japan), and the solution was lyophilized, followed by incubation at 90 °C for 30 min (39). CML-BSA was prepared as described previously (26). In brief, 10 mg/ml of BSA was incubated at 37 °C for 24 h with





Figure 6. Quantification of sorbitol, furosine, and CML in the rat lens by LC-ESI-QTOF or LC-ESI-MS/MS. Changes in sorbitol (*A*), furosine (*B*), and CML (*C*) in rat lens proteins were measured by LC-ESI-MS/MS. *Nor*, normal group (n = 6); *DM*, diabetic group (n = 6). The data are presented as mean \pm S.D. #, p < 0.001, *versus* normal at 1 week (Bonferroni test). In addition, A, *, p < 0.05, DM at 1 week *versus* DM 2 weeks. *B*, #, p < 0.001, DM at 1 week *versus* DM at 2 weeks; DM at 2 weeks; DM at 2 weeks (Bonferroni test).

75 mM glyoxylic acid monohydrate (FUJIFILM Wako Pure Chemical., Inc., Osaka, Japan) and 0.45 M NaCNBH₃ in 0.2 M sodium phosphate buffer (pH 7.8), followed by dialysis against PBS. To prepare the fructose-modified amino acids (Fruacetyl-Lys, Fru-acetyl-Arg), 50 mM N^{α} -acetyl-lysine (Tokyo Chemical Industry Co., Ltd.) or N^{α} -acetyl-Arg (Sigma) were mixed with 0.37 M fructose, and the solution was lyophilized. The lyophilized sample was incubated at 90 °C for 30 min. These modified BSA or amino acids were then subjected to noncompetitive or competitive ELISA using Fru-P antibody.

Preparation of Fru-P antibody

To prepare a polyclonal antibody against Fru-P, 0.1 mg of Fru-BSA in 50% Freund's complete adjuvant was injected intradermally on a rabbit, followed by nine booster injections of 0.1 mg of Fru-BSA in 50% Freund's incomplete adjuvant. Serum obtained 10 days after the final immunization was precipitated with ammonium sulfate. The precipitated antiserum protein was subjected to further affinity column. BSA or Fru-BSA were coupled to HiTrap NHS-activated HP Column (HiTrap-NHS). Anti-BSA antiserum was removed from the antiserum by the BSA-coupled HiTrap-NHS. Anti-Fru-BSA antiserum was purified by Fru-BSA-coupled HiTrap NHS, Fru-P antibody, which reacted to Fru-BSA was obtained (40, 41). The reactivity of the Fru-P antibody against Fru-BSA was measured by ELISA.

Animal experiments

All animal experiments were approved by Tokai University (approval number: 181089) and complied with Guidelines for the Care and Use of Animals for Scientific Purposes at Tokai University (established April 1, 2007). Wistar rats were purchased from Kyudo (Kumamoto, Japan). The rats were housed in a pathogen-free barrier facility (12-h light/dark cycle) and were fed a normal rodent chow diet (Clea, Tokyo, Japan). The rats were randomly divided into six groups (n = 6 per cage). Diabetes was induced in 6-week-old male rats (body weight ~150 g) by a single intravenous (tail vein) injection of STZ (50 mg/kg body weight) in 0.2 ml of 0.05 M saline-citrate buffer (pH 4.5). Diabetic rats (at 1, 2, 4, and 8 weeks after STZ injection) and nondiabetic rats (at 1 and 8 weeks after breeding start) were killed by decapitation under anesthesia with isoflurane. Serum samples were collected and analyzed to determine the blood glucose levels by Glucose CII-testwako (FUJIFILM Wako Pure Chemical) and HbA1c levels were determined by DCA vantage (SIEMENS Healthineers, Erlangen, Germany). The tissue specimens were immediately frozen and stored at -80 °C until analysis.

Detection of fructose-modified protein by Western blotting using Fru-P antibody

For the detection of Fru-BSA by Western blot, whole lens proteins (10 μ g) were subjected to 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes under semi-dry conditions using a Trans-blot (Bio-Rad). The membrane was blocked by incubation with 5% BSA in Tris-buffered saline (TBS; 1 mM Tris-HCl, pH 7.4, and 0.15 M NaCl) at room temperature for 1 h with gentle agitation, and washed three times with TBS containing 0.05% Tween 20 for 5 min each time. The membrane was incubated with 0.5 μ g/ml of Fru-P antibody at 4 °C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated streptavidin diluted to 1:5000 for 1 h at room temperature and then washed three times with TBS containing 0.05% Tween 20 for 5 min each time. The chemiluminescence method was employed to amplify the





Figure 7. Quantification of GL in the rat lens by LC-ESI-MS/MS. *A*, typical chromatogram of GL and $[{}^{13}C_{6}]$ GL in the rat lens obtained by LC-ESI-MS/MS. Changes in GL (*B*) in rat lens proteins were measured by LC-ESI-MS/MS. The variability in the rate of CML, furosine, and GL are indicated (*C*). *Nor*, normal group (n = 6); *DM*, diabetic group (n = 6). The data are presented as mean \pm S.D. $\pm p < 0.01$, *versus* normal 1 week (Student's t test); #, p < 0.001, *versus* normal at 1 week (Bonferroni test). In addition, *B*, #, p < 0.001, DM at 1 week *versus* DM at 2 weeks; DM at 2 weeks *versus* DM at 4 weeks; DM at 4 weeks *versus* DM at 8 weeks (Bonferroni test).

signal using an Amersham Biosciences ECL Prime system (GE Healthcare) (24). The density of detected bands in each lane was measured using Image Gauge software in LAS-3000 mini (FUJIFILM, Tokyo, Japan). The part on the gel outside the lanes was utilized as control, where no protein was applied.

ELISA

ELISAs were performed as described previously (20, 42). In brief, for noncompetitive ELISA, each well of a 96-well immunoplate (Thermo Fisher Scientific) was coated with 0.1 ml of the sample in PBS and blocked with 0.5% gelatin hydrolysate in PBS. The wells were incubated for 1 h with 0.1 ml of the 0.5 μ g/ml of the Fru-P antibody or CML antibody. The antibody bound to wells was detected by horseradish peroxidase-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific). For competitive ELISAs, each well of the 96-well immunoplate was coated with 0.1 ml of 0.01 μ g/ml of Fru-BSA in PBS and blocked with 0.5% gelatin hydrolysate in PBS. After addition of 50 μ l of the sample, the same volume of the Fru-P antibody solution was added to each well. The antibody bound to wells was detected by horseradish peroxidase-conjugated anti-mouse IgG antibody.

Purification of the epitope structure of Fru-P antibody

To prepare fructose-modified N^{α} -Cbz-lysine (Fru-Cbz-Lys), 70 mM N^{α} -Cbz-lysine (Sigma) was mixed with 0.37 M fructose, and the solution was lyophilized. The lyophilized sample was incubated at 90 °C for 30 min. For preparative HPLC (SHIMAZDU, Kyoto: system controller, CBM-20A; pump, LC-20AD; UV detector, SPD-20A; autosampler, SIL-20A; column oven, CTO-20AC), 30 µl of the Fru-Cbz-Lys was injected into the Cosmosil Packed column 5C₁₈-AR-II (20 imes 250 mm; Nacalai Tesque Inc., Kyoto, Japan). The column was maintained at 40 °C. The mobile phase was 0.1% TFA (FUJIFILM Wako Pure Chemical, Inc.), with a two-step gradient of acetonitrile (ACN) (0-2.5 min, 10-25% ACN; 2.5-20 min, 25-47.5% ACN; 20-22.25 min, 47.5-60% ACN; 22.25-40 min, 60% ACN) with a flow rate of 3 ml/min. Effluents were monitored for UV of 270 nm and separated into four fractions (fraction 1, 21–22.5 min; fraction 2, 24.5–25.5 min; fraction 3, 27-28.5 min; fraction 4, 37.5-39 min). These fractionations were repeated 20 times, and each fraction was lyophilized. The lyophilized samples were reconstituted in water, and aliquots of each fraction were subjected to competitive ELISA using Fru-P



antibody as described above. Fraction 2 was confirmed to contain the Fru-P antibody epitope by competitive ELISA. To further separate fraction 2, Fru-Cbz-Lys was applied to an HPLC system with a different gradient condition. The mobile phase was 0.1% TFA with a two-step gradient of ACN (0-3 min, 10% ACN; 3-5 min, 10-25% ACN; 5-25 min, 25-27.5% ACN; 25-35 min, 27.5-40% ACN; 35-40 min, 40-60% ACN). Eluents were monitored for UV of 270 nm and separated into two fractions (fraction 2-1, 34-36 min; fraction 2-2, 36-38 min). These fractionations were repeated 20 times, and each fraction was subjected to competitive ELISA using Fru-P antibody. Fraction 2-1 was then subjected to NMR and LC-ESI-QTOF. The Cbz group of fraction 2-1 was deprotected by catalytic reduction using 1 ml of methanol containing 5% palladium carbon (Nacalai Tesque Inc., Kyoto, Japan) under a hydrogen atmosphere (43).

NMR structural analysis

Fraction 2-1 was characterized by NMR using a DRX-500 spectrometer equipped with a cryogenic TXI probe (Bruker BioSpin, Billerica, MA). The probe temperature was set to 298 K. The sample (fraction 2-1, 5 mg) was dissolved in 600 μ l of D_2O (99.99 atom% D), and the ¹H chemical shifts were reported relative to the external standard of 4,4-dimethyl-4-silapentane-1-sulfonic acid. The ¹³C- and ¹⁵N-chemical shifts were calibrated using an indirect reference based on the X/¹H resonance ratio of 0.251449530 (13C/1H) and 0.101329118 (15N/1H). NMR signals were assigned by 1D ¹H, 1D-selective TOCSY, 1D-selective NOESY, 1D ¹³C, 2D ¹H-¹H DQF-COSY, ¹H-¹³C HSQC, ¹H-¹³C HSQC-TOCSY, ¹H-¹³C HSQC-NOESY, ¹H-¹³C HSQC-ROESY, ¹H-¹³C HMBC, and ¹H-¹⁵N HMBC. Data processing and analysis were performed using XWIN-NMR (version 3.5, Bruker BioSpin). NMR spectra were displayed with XWIN-PLOT (version 3.5, Bruker BioSpin).

Structural analysis by LC-ESI-QTOF

The pooled elution of fraction 2-1 was dried and resuspended in 20% ACN containing 0.1% formic acid (FA). The accurate mass of fraction 2-1 was determined by LC-ESI-QTOF using a compact mass spectrometer (Bruker Daltonics, Bremen, Germany). The mobile phase was an isocratic flow of 80% ACN containing 0.1% FA. The flow rate was set to 0.2 ml/min and the injection volume was 5 µl. Instrument calibration was performed externally for each assessment with 50% 2-propanol containing 5 mM sodium formate. The samples were analyzed at flow injection by ESI-positive MS multiple reaction monitoring (MRM). The ionization source temperature was 200 °C, and the capillary voltage was 4.5 kV. Collision-induced dissociation was performed using nitrogen with the collision energy set to 20 eV and pressure of 1.6 bar. Data were acquired with a stored mass range of m/z 50-1000. The composition formula of detected ions was analyzed by SmartFormula manually (Bruker Daltonics, Bremen, Germany) (14).

Measurement of CML, GL, furosine in vitro, and CML in the rat lens by LC-ESI-QTOF

LC was conducted on a ZIC[®]-HILIC column (2.1 \times 150 mm, 5 μ m; Merck Millipore, Billerica, MA, USA) maintained at

Identification of glucoselysine in diabetic lens

Table 3

Retention time and *m/z* values of AGEs, Amadori product, and amino acids determined by LC-ESI-QTOF

Analyte	Retention time	Parent ion		
	min	m/z		
CML	14.1	205.1183		
[² H ₂]CML	14.1	207.1308		
GL	15.7	309.1656		
[¹³ C ₆]GL	15.7	315.1858		
Furosine	13.5	255.1339		
[¹³ C ₆]Furosine	13.5	261.1541		
Lysine	15.8	147.1128		
[¹³ C ₆]Lysine	15.8	153.1329		

40 °C. The mobile phase was 0.1% FA (pH 3.8), with a two-step gradient of ACN (0–2 min, 90% ACN; 2–16 min, 90–10% ACN; 16–19 min, 10% ACN). The flow rate was set to 0.2 ml/min and the injection volume was 5 μ l. GL, CML, furosine, lysine, and their internal standards were detected by ESI-positive MS. The [²H₂]CML standard was purchased from Poly-Peptide Laboratories (Strasbourg, France), and the [¹³C₆]lysine standard was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). The retention time and quantitative ions are indicated in Table 3.

Measurement of sorbitol in the rat lens by LC-ESI-QTOF

LC was conducted on a ZIC[®]-HILIC column maintained at 40 °C. The mobile phase was 10 mM ammonium acetate, with an isocratic flow of 80% ACN. The flow rate was set to 0.2 ml/min and the injection volume was 5 μ l. Sorbitol and its internal standard were detected by ESI-negative MS. The D-[¹³C₆]sorbitol standard was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). The retention times of sorbitol and [¹³C₆]sorbitol were 5.9 min, respectively. The quantitative ions of sorbitol and [¹³C₆]sorbitol were *m*/*z* 181.0707 and 189.0908, respectively.

Measurement of GL and furosine in the rat lens and kidney by LC-ESI-MS/MS

LC was conducted using the same conditions as described above for the measurement of AGEs or Amadori product by LC-ESI-QTOF. The accurate masses of GL and furosine were determined by LC-ESI-MS/MS using a TSQ Quantiva system (Thermo Fisher Scientific) with ESI-positive MRM. The flow rate was set to 0.2 ml/min and the injection volume was 10 μ l. A traditional ESI probe was used to quantify GL and furosine in the rat lens, and the parameters were optimized as follows: nitrogen sheath gas, 35 Arb; auxiliary gas, 15 Arb; spray voltage, 3.5 kV; ion transfer tube temperature, 270 °C; and vaporizer temperature, 250 °C. Argon gas was used as the collision gas and the pressure was set at 2.0 mTorr. GL content in lens and kidney was measured by quantitative ion, m/z 210.1 $(C_{11}H_{16}NO_3)$, which is one of the fragment ions of GL as shown in Table 2. The retention time and quantitative ions are indicated in Table 4 (44).

Chemical properties of GL and FL

For evaluation of the stability of GL or FL under acid hydrolysis, 10 pmol of GL or FL standard was hydrolyzed with 1 ml of 6 $_{\rm M}$ HCl at 100 °C for 0–48 h. The pooled samples were dried

Table 4

Retention time and *m/z* values of AGEs, Amadori products, and amino acids by LC-ESI-MS/MS

Analyte	Retention time	Collision energy	Parent ion	Fragment ion	
	min	eV		m/z	
GL	15.7	18.8	309.2	210.1	
[¹³ C ₆]GL	15.7	19.0	315.2	216.1	
Furosine	13.5	15.1	255.1	192.1	
[¹³ C ₆]Furosine	13.5	15.7	261.2	198.1	
Lysine	15.8	15.0	147.1	84.0	
[¹³ C ₆]Lysine	15.8	15.0	153.1	89.0	

and resuspended in 100 μl of 20% ACN containing 0.1% FA. The samples were then subjected to LC-ESI-QTOF.

For formation of GL and FL under physiological condition, BSA was reduced with 0.1 M NaBH₄ and 0.2 M sodium borate buffer (pH 9.1) at room temperature for 4 h. The RdBSA was then dialyzed against 0.2 M sodium phosphate buffer (pH 7.4) at 4 °C for 18 h, and the protein concentration was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). RdBSA (1.5 mg/ml) was incubated with 30 mM fructose (Fru-RdBSA), glucose (Kanto Chemical Co., Inc.; Glu-RdBSA), galactose (FUJIFILM Wako Pure Chemical., Inc., Osaka, Japan; Gal-RdBSA), or mannose (Kanto Chemical Company Inc.; Man-RdBSA) at 37 °C for 0-14 days in 0.2 M sodium phosphate buffer (pH 7.4). The samples were dialyzed against diluted water at 4 °C for 18 h, and the protein concentrations were measured by the BCA assay. Ten micrograms of modified BSA samples were hydrolyzed with 1 ml of 6 M HCl at 100 °C for 18 h. The pooled samples were dried and resuspended in 50 μ l of 20% ACN containing 0.1% FA. The samples were then subjected to LC-ESI-QTOF.

Conversion of GL or FL to CML under oxidative stress

GL or FL (20 pmol) was incubated 37 °C for 1 h with 0.4 mM FeCl₂ in the absence or presence of 0.1 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.2). The sample solutions were added to 1 ml of 0.1% TFA and passed over a Strata-X-C column (Phenomenex, Torrance, CA), which had been prewashed with 1 ml of methanol and equilibrated with 1 ml of 0.1% TFA. The samples were loaded to the column and the column was washed with 3 ml of 2% FA and eluted with 1.5 ml of 20% methanol containing 7% ammonia. The pooled elution fractions were dried and resuspended in 0.2 ml of 20% ACN containing 0.1% FA (21). The samples were then subjected to LC-ESI-QTOF.

Preparation for measurement of AGEs and Amadori product in the rat lens and kidney

The rat lens was homogenized with 0.2 ml of 1 mM diethylenetriamine-N,N,N',N'',N''-pentaacetic acid (Dojindo Laboratories, Kumamoto, Japan) by Shake master auto version 2 (Biomedical Science, Tokyo, Japan). The kidneys, cut into several pieces, removed blood by gentle agitation at 4 °C for 72 h in PBS. The rat kidney was homogenized with 5 ml of 0.5% CHAPS/PBS (Dojindo Laboratories, Kumamoto, Japan) by Polytron PT 10–35 GT (Kinematica, Luzern, Swiss). The protein concentrations of homogenized samples were measured by the BCA method.

Preparation for measurement of CML in the rat lens

Rat lens samples (0.3 mg each) were used for measurement of CML. The samples were reduced with NaBH₄ (2 μ l of 1 M NaBH₄ in 0.1 N NaOH) in 20 μ l of 200 mM sodium borate buffer (pH 9.1) at room temperature for 4 h. CML and lysine internal standards were added to the pellets, which were hydrolyzed with 1 ml of 6 M HCl at 100 °C for 18 h. The dried sample was resuspended in 1 ml of 0.1% TFA and passed over a Strata-X-C column, which had been pre-washed with 1 ml of methanol and equilibrated with 1 ml of the solution, similar to the sample solvent, and loaded to the column. The column was then washed with 2% FA and eluted with 20% methanol containing 7% ammonia. The pooled elution fractions were dried and resuspended in 0.2 ml of 20% ACN containing 0.1% FA. The samples were then subjected to LC-ESI-QTOF.

Preparation for measurement of GL and furosine in the rat lens and kidney

Rat lens or kidney samples (0.3 or 0.2 mg each) were used for measurement of GL and furosine. GL, furosine, lysine, and their internal standards were added to the samples, which were hydrolyzed with 1 ml of 6 mmm HCl at 100 °C for 18 h. The dried sample was resuspended in 1 ml of 0.1% TFA and filtered through a Sep-Pak C18 column, which had been pre-washed with 1 ml of methanol and equilibrated with 1 ml of the solution similar to the sample solvent. The pooled flow-through fractions and 1 ml of the same solution fractions were dried and resuspended in 1 ml of 20% ACN containing 0.1% FA. The samples were then subjected to LC-ESI-MS/MS (44, 45).

Preparation for measurement of sorbitol in the rat lens

One milligram of rat lens samples were used for measurement of sorbitol. The solution volume was adjusted to 0.2 ml with 1% TFA. The sorbitol internal standard was added to the solutions, which were filtered by a 10,000 molecular weight cut-off filter. The filtered solutions (0.1 ml each) were added to 0.9 ml of 1% TFA and passed over a Sep-Pak C18 column, which had been pre-washed with 1 ml of methanol and equilibrated with 1 ml of 1% TFA, and then loaded to the column. The pooled flow-through fractions and 1 ml of the same solution fractions were dried and resuspended in 0.1 ml of 20% ACN. The samples were then subjected to LC-ESI-QTOF.

Statistical analysis

Data were expressed as mean \pm S.D. (Table 1, Figs. 1 and 5–7). The differences in the levels of GL, CML, sorbitol, and furosine between the groups were examined for statistical significance using the one-way analysis of variance (normal, 1 week *versus* normal 8 weeks; DM, 1, 2, 4, and 8 weeks). Student's *t* test and subsequent post hoc analysis (Bonferroni correction method) were used to correct for multiple comparisons.

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Identification of glucoselysine in diabetic lens

16. Ni, J., Yuan, X., Gu, J., Yue

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Identification of glucoselysine in diabetic lens

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Glucoselysine is derived from fructose and accumulates in the eye lens of diabetic rats

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