Increased Levels of Advanced Glycosylation End Products in the Kidney and Liver From Spontaneously Diabetic Chinese Hamsters Determined by Immunochemical Assay

Atsuko Abiko, Masaaki Eto, Isao Makino, Norie Araki, and Seikoh Horiuchi

Increased levels of advanced glycosylation end products (AGEs) have been reported in tissues in association with diabetes mellitus. Thus, we measured tissue AGE levels and detected an accumulation of AGEs in the kidney and liver from spontaneously diabetic Chinese hamsters (CHAD) to determine the relationship between AGEs and diabetes mellitus. Diabetic CHAD aged 12 to 13 months were studied together with age-matched nondiabetic CHAD. We used an AGE-specific noncompetitive enzyme-linked immunosorbent assay (ELISA) with polyclonal anti-AGE-bovine serum albumin (BSA) antibody to measure tissue AGE levels. The samples extracted from the kidney and liver obtained from diabetic and nondiabetic CHAD reacted with anti-AGE-BSA antibody. When the absorbance of standard AGE-BSA (0.1 μg/mL) was expressed as 1 U, AGE levels in the kidney and liver from diabetic CHAD were significantly increased as compared with nondiabetic CHAD (kidney, 0.26 ± 0.05 *v* 0.10 ± 0.03 U/μg protein, $P < .01$; liver, 0.20 ± 0.03 *v* 0.09 ± 0.02 U/μg protein, $P < .01$). Positive AGE staining was observed in the renal cortex, especially in the tubules of diabetic CHAD, but little AGE staining was observed in the glomerulus by the immunohistochemical study. AGE staining was diffuse in the hepatocytes. These AGE levels were significantly correlated with fasting plasma glucose and glycated hemoglobin ($P < .01$, respectively). In conclusion, we have confirmed that AGE structures were expressed in the kidney and liver from CHAD, and these AGE levels were increased in diabetic CHAD. AGE staining was observed in the renal tubules and hepatocytes. Tissue AGE levels were positively correlated with glycemic control in CHAD.

THE ADVANCED Maillard reaction is a posttranslational modification process that results from the spontaneous nonenzymatic reaction of circulating glucose with protein amino groups. The chronic hyperglycemia of diabetes mellitus increases the formation of advanced glycosylation end products (AGEs).[1-3] AGEs have been proposed to play a role in the pathogenesis of diabetic complications and in the aging process.[1-3] The main features of AGEs such as fluorescence, a brown color, cross-linking, and biological recognition are generally known,[1-5] but the main chemical structures and biological significance of these products remain unknown. Recently, several chemical structures and measurement methods for AGEs have been proposed. In the experimental approaches, AGEs that have been prepared in vitro have been used to elicit AGE-specific monoclonal and polyclonal antibodies.[6-8] AGEs that are formed in vivo in collagen,[9,10] hemoglobin,[11] and lens crystalline[12,13] were detected by immunochemical assay using these anti-AGE antibodies. In the present study, we used polyclonal antibodies that did not recognize FFI, pentosidine, and pyrraline but did recognize the structures commonly expressed by AGE structures.[6,12] In addition, our recent study demonstrated that these polyclonal antibodies are of 2 types, one specific for a *N*-(carboxymethyl)lysine protein (CML)-protein adduct and the other specific for the non-CML AGE structures but still common to AGE structures.[14] AGE levels are increased with chronic hyperglycemia or aging, but little information is available about the relationship between AGEs and diabetic complications.

Spontaneously diabetic Chinese hamsters of the Asahikawa colony (CHAD) are a strain predisposed to diabetes mellitus by continuous brother-sister inbreeding.[15,16] The onset of diabetes in CHAD occurs within 3 months, and these animals are non-obese and live more than 1 year after the onset of hyperglycemia without exogenous insulin.[17,18] Hyperinsulinemia and peripheral insulin resistance precede hyperglycemia.[19] CHAD with overt diabetes show hypoinsulinemia, a decreased β-cell number in the islets, and a low insulin response to glucose.[17,20,21] These characteristics of CHAD are similar to some types of type 2 diabetes in humans, and thus CHAD have been used to investigate the pathogenesis of type 2 diabetes and diabetic complications. With regard to renal dysfunction of CHAD, age-dependent increases of urinary protein excretion, renal and glomerular hypertrophy, increases of mesangial matrix, and thickening of glomerular basement membrane have been reported.[22] The pathological changes of the kidney do not result in glomerular sclerosis, but some findings in CHAD are similar to early diabetic nephropathy in humans.

The kidney is a main tissue in which diabetic complication occurs, and the liver is an important tissue which regulates glucose metabolism. Thus, in the present study, we measured tissue AGE levels in CHAD by an immunochemical method and detected the accumulation of AGEs in the kidney and liver from CHAD by an immunohistochemical study with anti-AGE antibody. Moreover, we determined the relationship between tissue AGE levels and the diabetic state.

MATERIALS AND METHODS

Animals

Diabetic CHAD that showed positive urine glucose aged 12 to 13 months and age-matched nondiabetic CHAD were used in this experiment. The animals were housed at 25°C with illumination from 5 AM to 7 PM. They were fed normal laboratory chow (MF; Oriental Kobo, Tokyo, Japan) and had free access to water. After an overnight fast (16 to 17 hours), blood samples were obtained from the jugular vein of the

From the Second Department of Internal Medicine, Asahikawa Medical College, Asahikawa; and Second Department of Biochemistry, Kumamoto University Medical School, Kumamoto, Japan.

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Address reprint requests to Masaaki Eto, MD, Kawasaki Medical School, Department of Medicine, Diabetes Division, Matsushima 577, Kurashiki 701-0192, Japan.

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animals anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), and the plasma was kept at −20°C until assay. Then the organs were removed under laparotomy. The organs were washed with saline, immediately frozen by dry ice in acetone, and kept at −40°C until assay. Part of each organ was embedded in Tissue-Tek ornithine carbamoyltransferase (Miles, Elkhart, IN) compound and snap-frozen at −40°C for immunohistochemical study. The rest was immersed in 10% formaldehyde solution for morphological study. These studies were approved by the Asahikawa Medical College Institutional Animal Care Committee.

Reagents

Bovine serum albumin (BSA fraction V) was purchased from Sigma (St Louis, MO). AGE-BSA and polyclonal anti-AGE antibody were obtained from the Second Department of Biochemistry, Kumamoto University Medical School. They were produced and characterized as described previously.[6,12,14] AGE-BSA was made as follows: 1.6 g BSA was dissolved with 3.0 g D-glucose in 10 mL 0.5-mol/L sodium phosphate buffer, pH 7.4, and incubated at 37°C for 90 days. Peroxidase-conjugated swine anti-rabbit immunoglobulin was purchased from Dako (Glostrup, Denmark). For immunohistochemistry, 10% normal rabbit serum and peroxidase-conjugated streptavidin were used from the HISTOFINE SAB-PO kit (Nichirei, Tokyo, Japan). Streptavidin was purchased from Wako (Tokyo).

Preparation of Organic Samples

The kidney and liver obtained from CHAD were homogenized in 500 mg weight per 1 mL 0.1N NaOH on ice and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was removed into a new tube. The precipitate was repeatedly homogenized in an equal volume of 0.1N NaOH and centrifuged at 14,000 rpm for 15 minutes at 4°C, and the supernatant was added into the tube. The mixture of the supernatant, ie, the alkaline-soluble sample, was used for AGE measurement. Each sample was adjusted to 1 mg/mL protein by dilution with 0.1N NaOH and kept at −20°C until analysis.

Enzyme-Linked Immunosorbent Assay

AGE measurements were performed in a noncompetitive ELISA as described previously.[6,12] All processes were performed at room temperature. A 96-well microtiter plate (Nunc-Immunoplate; GIBCO, Grand Island, NY) was coated with 0.1 mL sample (10 mg/mL dissolved in 5 mmol/L carbonate buffer, pH 9.6) and incubated for 2 hours. The wells were washed 3 times with phosphate buffer solution (PBS) containing 0.05% Tween 20 (buffer A) and then blocked with 0.5% gelatin for 1 hour. After washing with buffer A, the wells were reacted with 0.1 mL polyclonal anti-AGE antibody diluted by buffer A with 0.1% BSA for 2 hours. Then, the wells were washed with buffer A, incubated with peroxidase-conjugated anti-rabbit antibody, and reacted with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by 1 mol/L sulfuric acid, and absorbance at 492 nm was read on a micro-ELISA plate reader (Titertek Multiskan Plus MK II; Flow Laboratories, Lugano). As the standard, AGE-BSA was dissolved in 5 mmol/L carbonate buffer, pH 9.6, and then measured by the same method. The absorbance of standard AGE-BSA (0.1 μg/mL) was determined as 1 U. The amount of AGEs in the samples was calculated by (absorbance at 492 nm of samples/absorbance at 492 nm of standard AGE-BSA) U/μg tissue protein. The concentration of anti-AGE antibody was 3 μg/mL. The intraassay CV was 6.7% and interassay CV 9.7%.

Immunohistochemical Procedures

The distribution of AGEs in the kidney and liver was studied with immunoperoxidase techniques by the labeled streptavidin-biotin method. Kidney and liver specimens were immediately snap-frozen, and sections 5 μm thick were prepared. Endogenous peroxidase activity was inhibited by a 20-minute incubation with methanol containing 0.6% H_2O_2. Nonspecific staining was blocked by a 10-minute incubation with streptavidin. Nonspecific protein binding sites were blocked by 10 minutes of incubation with 10% normal rabbit serum. Slides were stained with biotin-labeled rabbit anti-AGE polyclonal antibody overnight at 4°C. The slides were washed with PBS and incubated with peroxidase-conjugated streptavidin for 5 minutes. They were then washed in PBS and the color reaction was performed by incubation with aminoethylcalbasole reagent until staining was complete. The reaction was stopped by placing each slide in water, and then Mayer's hematoxylin was added as a counterstain. Negative control staining was performed using the same anti-AGE antibody preincubated with an excess of AGE-BSA.

Morphological Study

The kidney and liver in formaldehyde solution were embedded in paraffin and stained with periodic acid–Schiff (PAS) for light microscopic observation.

Analysis

Protein was assayed by the BCA method (BCA Protein Assay Reagent; Pierce, Rockford, IL). Fasting plasma glucose was measured by the glucose oxidase method (Diyacolor-GC; Toyobo, Osaka, Japan). Glycated hemoglobin was measured by boronate affinity chromatography (Glyc-Affin-GHb; Seikagakukogyo, Tokyo, Japan). Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels were measured by the enzyme method (Transaminase-CII-test-Wako; Wako).

Statistics

The data are expressed as the mean ± SD. Differences between the 2 groups were analyzed nonparametrically with the Mann-Whitney *U* test. Pearson's correlation coefficient (*r*) was used to assess the relationship of tissue AGE levels to glycated hemoglobin and fasting plasma glucose. A *P* level less than .05 was considered significant.

RESULTS

Characteristics of the Animals

Table 1 shows the characteristics of diabetic (n = 6) and nondiabetic (n = 5) CHAD. Body weight was not significantly different between the 2 groups. The kidney weight of diabetic CHAD was significantly increased compared with nondiabetic

Table 1. Characteristics of CHAD in This Study

Characteristic	Nondiabetic CHAD	Diabetic CHAD
No. of animals (male/female)	5 (1/4)	6 (1/5)
Body weight (g)	28.7 ± 5.4	29.3 ± 3.8
Kidney weight (mg)	128.0 ± 14.8	226.7 ± 57.6*
Liver weight (mg)	1,012.0 ± 171.7	1,682.0 ± 174.1*
Fasting plasma glucose (mg/dL)	73.7 ± 19.8	414.3 ± 86.9*
Glycated Hb (%)	5.7 ± 1.1	25.3 ± 2.9*
GOT (KU)	52.3 ± 12.5	89.2 ± 52.6
GPT (KU)	17.3 ± 4.5	12.8 ± 3.4

NOTE. The animals are aged 12 to 13 months. Data are the mean ± SD.

*$P < .01$, diabetic *v* nondiabetic CHAD.

CHAD (226.7 ± 57.6 *v* 128.1 ± 14.8 mg). Similarly, the liver weight of diabetic CHAD was significantly increased (1,682.0 ± 174.1 *v* 1,012.0 ± 171.7 mg). Diabetic CHAD had significantly higher levels of fasting plasma glucose and glycated hemoglobin than nondiabetic CHAD. The levels of GOT and GPT were not significantly different between the 2 groups.

Figure 1A shows a light micrograph of the renal corpuscle in diabetic CHAD. The glomerulus in diabetic CHAD showed hypertrophy and increased PAS-positive materials as compared with nondiabetic CHAD. Glomerulosclerosis was not found in these specimens. Figure 2A shows a light micrograph of the liver in diabetic CHAD. In diabetic CHAD, PAS-positive materials were distributed diffusely in hepatocytes, but no inflammatory change or fatty change was observed.

AGE Levels in the Kidney and Liver

The immunoreactivity versus the concentration of AGE-BSA as a standard is shown in Fig 3A. AGE-BSA 0.1 μg/mL was used to calculate AGE levels of the samples. The immunoreactivity of the samples and standard AGE-BSA versus the concentration of anti-AGE antibody is shown in Fig 3B. The alkaline-soluble samples of the kidney and liver obtained from CHAD reacted with anti-AGE antibody, suggesting that the kidney and liver from CHAD expressed common AGE structures. The reactivities increased as the antibody concentrations increased. The reactivities of diabetic CHAD were higher than those of nondiabetic CHAD.

The absorbance of standard AGE-BSA (0.1 μg/mL) was determined as 1 U when the concentration of anti-AGE antibody was 3 μg/mL. AGE levels of the kidney and liver are shown in Fig 4. The kidney AGE level of diabetic CHAD was significantly increased as compared with nondiabetic CHAD (0.26 ± 0.05 *v* 0.10 ± 0.03 U/μg protein, $P < .01$). The liver AGE level of diabetic CHAD was also significantly increased (0.20 ± 0.03 *v* 0.09 ± 0.02 U/μg protein, $P < .01$).

Immunohistochemical Localization of AGEs

AGEs were stained in the renal cortex specimens from diabetic CHAD (Fig 1B). There was no staining in the negative control. AGE staining was strong in tubular cells and part of Bowman's capsule, but was not observed in the glomerulus (Fig 1C). In nondiabetic CHAD, AGE staining was weak in the renal cortex.

In the liver, AGE staining was diffuse in the hepatocytes, although it was not clear which component was stained (Fig 2B). AGE staining in diabetic CHAD was stronger than that in nondiabetic CHAD.

Correlation Between AGE Levels and Biochemical Parameters of Diabetic Control

Statistical analysis of the data using Pearson's correlation test yielded a high correlation between AGE levels and biochemical parameters of glycemic control (Figs 5 and 6). The kidney and liver AGE levels were significantly correlated with fasting plasma glucose levels (kidney, $r = .879$, $P < .001$; liver, $r = .762$, $P < .01$). The kidney and liver AGE levels were significantly correlated with glycated hemoglobin levels (kidney, $r = .942$, $P < .001$; liver, $r = .839$, $P < .01$). If we use

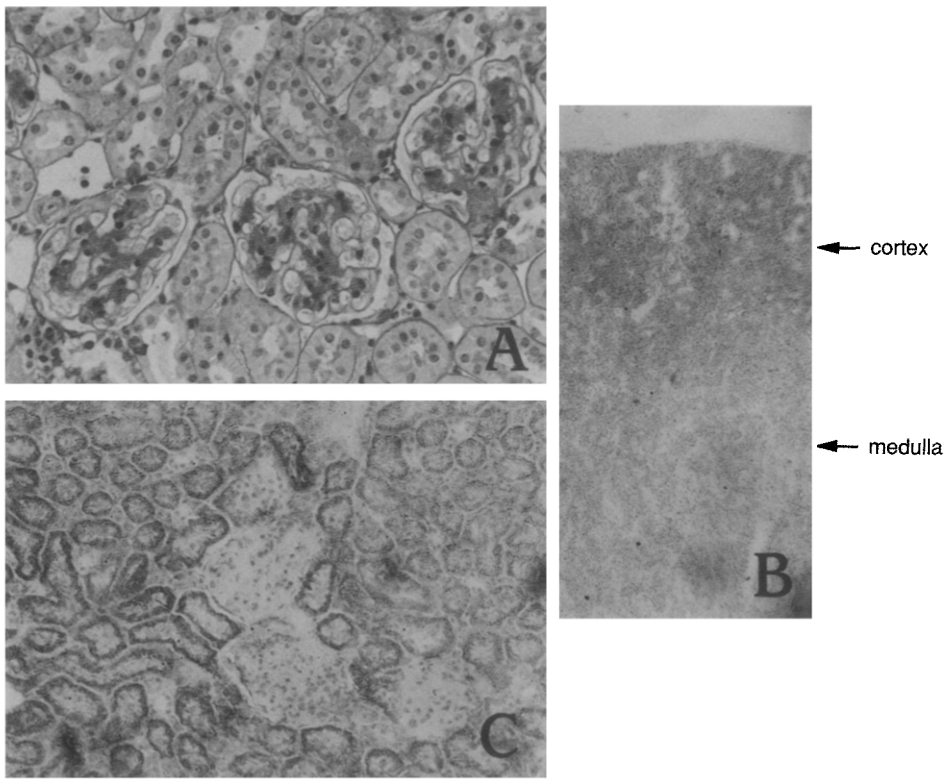

Fig 1. (A) Light micrograph of the glomerulus stained by PAS (×300). Diabetic CHAD show hypertrophy of the glomerulus and increased PAS-positive materials in the mesangial matrix. (B) ×30 and (C) ×150: Immunohistochemical detection of AGEs in the kidney of diabetic CHAD. Positive AGE staining is shown in the renal cortex (B), especially in the tubular cells and part of Bowman's capsule (C), but it is not shown in the glomerulus.

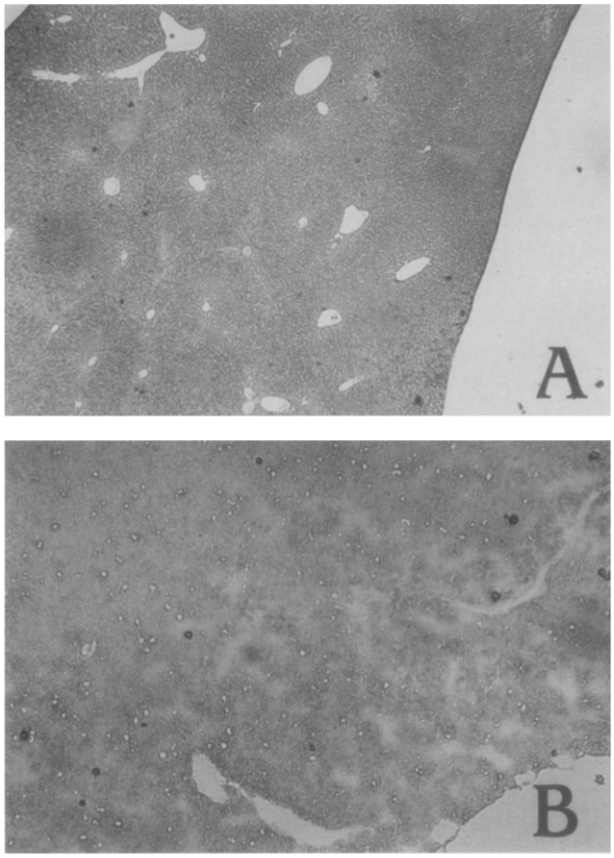

Fig 2. (A) Light micrograph of the liver stained by PAS (×30). PAS-positive materials are shown in the hepatocyte of diabetic CHAD. (B) Immunohistochemical detection of AGEs in the liver of diabetic CHAD (×30). Positive AGE staining is shown diffusely in the hepatocyte and is strong in diabetic CHAD.

Spearman's rank correlation test to exclude the clustering effect, the coefficients were greater than .8 and the *P* values were less than .05.

DISCUSSION

Recently, Horiuchi and Araki et al,[6,12] our colleagues in this study, prepared polyclonal and monoclonal anti-AGE antibodies against AGE-BSA and established an immunochemical (ELISA) method for AGE assay. The anti-AGE antibodies reacted specifically with AGE products, but not with unmodified proteins or early-stage products of the Maillard reaction. In addition, it was shown that the polyclonal antibodies are of 2 types, one specific for a CML-protein adduct and the other specific for the non-CML AGE structures but still common to AGE structures.[14] This immunochemical method using these antibodies demonstrated that common structures are present among AGE products derived from various proteins, that human lens crystallines contain AGEs, and that these AGEs increase with aging.[12] Therefore, AGE-specific immunochemical assay is a very sensitive and reliable method for detection of AGEs.

In the present study, we determined AGE levels in organs obtained from CHAD, a spontaneously diabetic animal model of type 2 diabetes, by this immunochemical assay using the polyclonal antibodies[6,12,14] to elucidate the relationship between AGEs and diabetes mellitus. The kidney and liver reacted with this antibody, indicating that common AGE structures were certainly expressed on organs from CHAD. AGE levels in the kidney and liver from diabetic CHAD aged 12 to 13 months were about 2 times higher than those from age-matched nondiabetic CHAD. Immunohistochemical study also showed the existence of AGEs in the kidney and liver.

The reasons that CHAD were used for the study of AGEs and diabetes mellitus are as follows. First, diabetic CHAD show severe hyperglycemia after the onset of diabetes mellitus.[16-21] In this study, fasting plasma glucose concentrations of diabetic CHAD were more than 400 mg/dL. Second, diabetic CHAD are able to live over 12 months without exogenous insulin infusion.[16-21] Hypoinsulinemia was caused by a reduction of β-cell numbers in the islets and pancreatic insulin content, but basal insulin secretion was not completely deficient in this animal.[16,19-21] Third, diabetic CHAD have renal damage that resembles early diabetic nephropathy in humans.[22] In the present study, we demonstrated that AGE levels in the kidney from diabetic CHAD were increased. The major factors that govern the formation of AGEs are the level of glucose and the duration of exposure to glucose. As stated before, diabetic CHAD in the present study were exposed to severe hyperglycemia for 9 to 10 months. Thus, there is a possibility that hyperglycemia causes excessive glycosylation of proteins and formation of AGEs, and AGEs may contribute to the pathological changes of the kidney by chemical modification and alteration of tissue proteins and then cause tissue functional disorder. Some reports suggest that AGEs are related to diabetic complications. Nakayama et al[9,13] reported that AGE levels of the lens, aorta, and renal cortex from streptozotocin (STZ)-induced diabetic rats were much higher than those from age-matched control rats. Recently, Beisswenger et al[10] reported that skin AGE levels correlated with early manifestations of renal and retinal diseases in patients with type 1 diabetes. In addition, aminoguanidine, an inhibitor of AGE formation, prevented the morphological changes and the increases in albuminuria and collagen-related fluorescence in the kidney of STZ-induced diabetic rats,[23-25] which supports the idea that AGEs may be part of the etiology of diabetic nephropathy. Therefore, we expected that AGEs would be involved in the renal damage of CHAD.

Furthermore, the present immunohistochemical study showed the accumulation of AGEs in the renal tubules of diabetic CHAD. There are prior immunohistochemical studies in humans. Yamada et al[26] reported positive AGE staining in the glomerulus and diffuse staining in renal tubular cells of early overt diabetic nephropathy. Makino et al[27] also reported positive AGE staining in the glomerular mesangium and tubules of diabetic nephropathy and an increase in the intensity with the progression of glomerulosclerosis. In the present study, contrary to our expectations, no glomerular staining for AGEs was observed in a specimen from diabetic CHAD. Therefore, AGEs

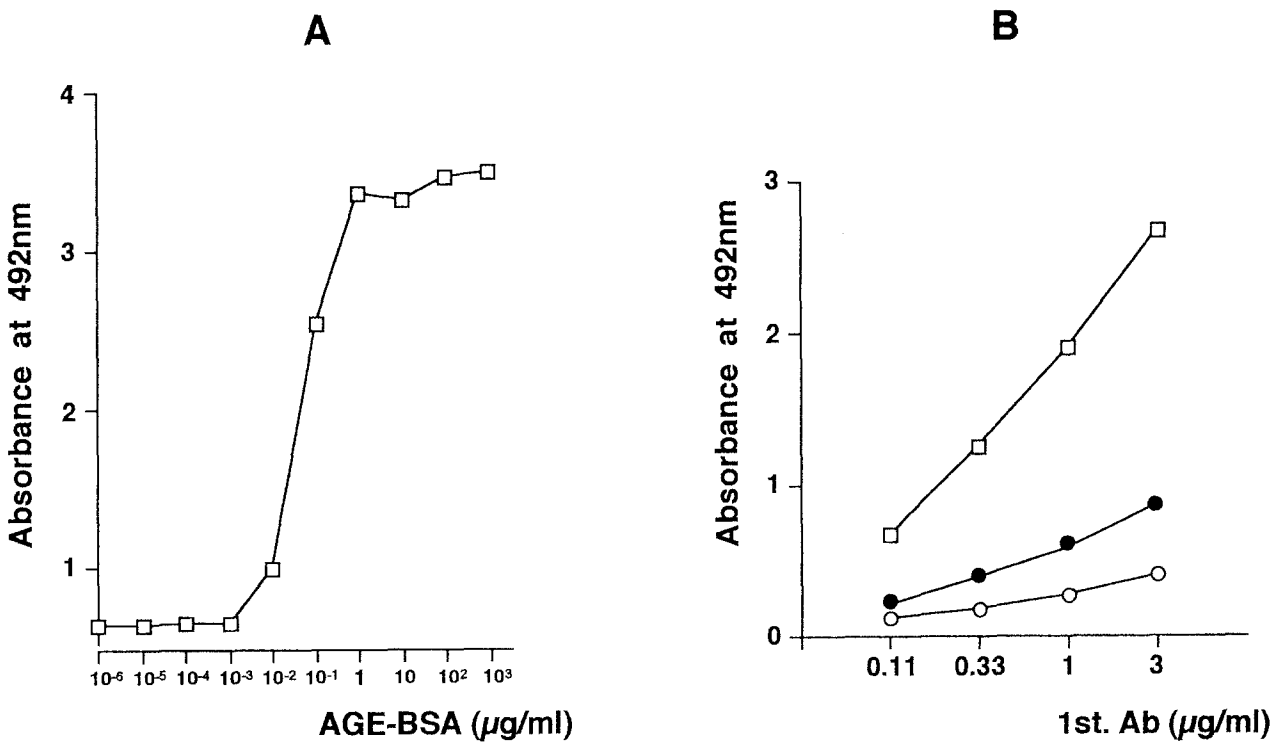

Fig 3. (A) Immunochemical reactivity versus the concentration of AGE-BSA as a standard to the polyclonal anti-AGE antibody. The concentration of anti-AGE antibody was 3 μg/mL. (B) Immunochemical reactivity of the samples and standard AGE-BSA versus the concentration of anti-AGE antibody. The concentration of standard AGE-BSA was 0.1 μg/mL. The amount of protein in each sample was fixed at 1 μg/well. Data are the mean ± SD. □, standard AGE-BSA; ○, kidney sample of nondiabetic CHAD; ●, kidney sample of diabetic CHAD.

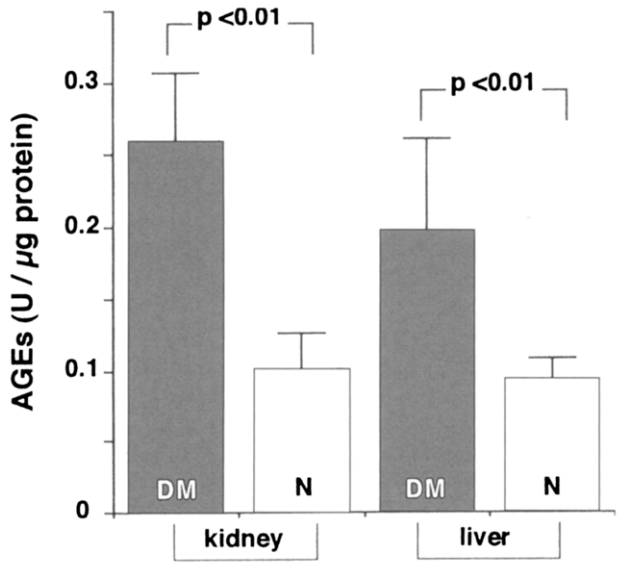

Fig 4. AGE levels in the kidney and liver obtained from CHAD. The concentration of anti-AGE antibody was 3 μg/mL and the amount of protein of each sample was fixed at 1 μg/well. The absorbance of standard AGE-BSA (0.1 μg/mL) was determined as 1 U. AGE levels are calculated by (absorbance at 492 nm of the sample/absorbance at 492 nm of standard AGE-BSA) U/μg tissue protein. Data are the mean ± SD. DM, diabetic CHAD (n = 6); N, age-matched nondiabetic CHAD (n = 5). DM *v* N, $P < .01$.

are not the PAS-positive materials in the glomerulus. This result might be attributed to the observation that glomerulosclerosis was not observed in the kidney of our diabetic CHAD. In humans, the duration of diabetes until the onset of diabetic nephropathy is more than 10 years. On the other hand, the average life span of CHAD is 1.5 to 2 years and the duration of diabetes is less than 2 years. The difference in the duration of diabetes between humans and CHAD is one possible explanation for the present observation that glomerulosclerosis and AGE staining in the glomerulus were not found in CHAD. Our anti-AGE antibodies did not stain the glomerulus in early diabetic nephropathy in humans (data not shown). Previous studies in humans[26,27] and the present study indicate that AGEs are present in renal tubules. The natural history of diabetic nephropathy is believed to reflect glomerular changes, but Bohle et al[28,29] emphasized the importance of tubulointerstitial changes rather than glomerular lesions in the progression of diabetic nephropathy. It also has been reported that tubular proteinuria and enzymuria, markers of tubular dysfunction, develop in the early stages of diabetic nephropathy in humans.[28-31] The mechanism and significance of the tubular change remains unknown, but the tubular deposition of AGEs may, at least in part, lead to a clue to the elucidation of tubular change in diabetic nephropathy. Further studies are needed.

Little is known about the relationship between the liver and AGEs in diabetes. It was reported that AGEs are recognized and endocytosed by sinusoidal liver cells,[32] and that the liver is a major site of AGE sequestration and isolated liver membrane

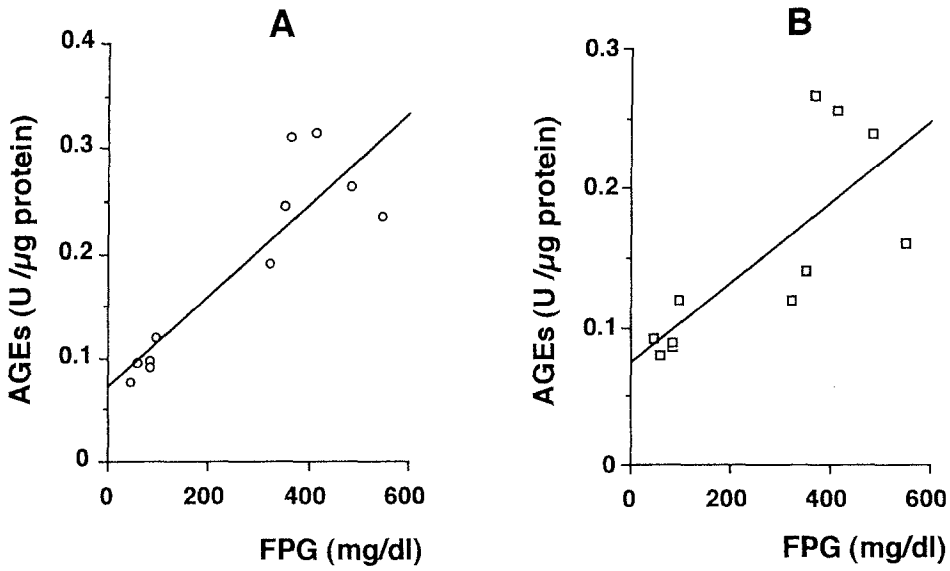

Fig 5. Correlation between AGE levels and fasting plasma glucose. (A) Kidney AGE levels; (B) liver AGE levels (n = 11). The regression equations in A and B are $r = .879$ ($P < .001$) and $r = .762$ ($P < .01$), respectively. Both data sets yield a high correlation between AGE levels and fasting plasma glucose.

proteins bind to AGEs.[33] Therefore, we investigated whether AGEs exist in the liver obtained from CHAD. We found that the liver from CHAD expressed common AGE structures, and AGE levels in the liver were increased in diabetic CHAD. AGE staining was observed in the hepatocytes. PAS-positive materials were increased in the liver from diabetic CHAD, indicating that carbohydrates such as glycogen were stored in the liver. We did not test which proteins in the liver are glycosylated and whether AGEs are the PAS-positive materials in the hepatocytes. The liver is not normally a site of diabetic complications. Further studies are needed to clarify the significance of the accumulation of AGEs in the liver of diabetic CHAD.

We also observed that AGE levels in the kidney and liver showed a positive correlation with fasting plasma glucose and glycated hemoglobin, which reflects glycemic control during the 6- to 8-week period before blood sampling, and that tissue AGE levels were more highly correlated with glycated hemoglobin than with plasma glucose, confirming a positive correlation between tissue AGE levels and long-term glycemic control. It is reasonable that tissue AGE levels should be positively correlated with glycated hemoglobin, because AGEs are produced by a nonenzymatic reaction of protein with glucose over a long period. However, there have been few studies on the relationship between AGEs and long-term glycemic control. Beisswenger et al[10,34] reported a positive relationship between skin AGEs and hemoglobin A_{1C} in patients with type 1 diabetes. It is suggested that AGEs may also reflect long-term glycemic control.

In conclusion, we have confirmed that the common AGE structures were expressed in the kidney and liver obtained from CHAD. We measured tissue AGE levels in CHAD by immunochemical assay and demonstrated that AGE in the kidney and

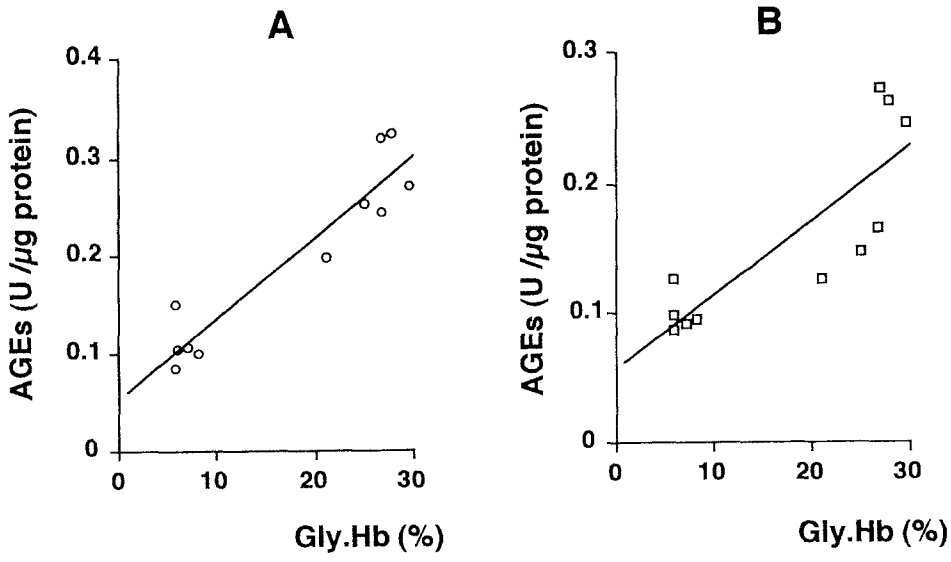

Fig 6. Correlation between AGE levels and glycated Hb. (A) Kidney AGE levels; (B) liver AGE levels (n = 11). The regression equations in A and B are $r = .942$ ($P < .001$) and $r = .839$ ($P < .01$), respectively. Both data sets yield a high correlation between AGE levels and glycated Hb.

liver obtained from diabetic CHAD were increased. Positive AGE staining was found in the renal tubules of diabetic CHAD in the immunohistochemical study. In the liver, positive AGE staining was observed in the hepatocytes. We also demonstrated a positive correlation between tissue AGE levels and long-term glycemic control in CHAD.

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